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Rotational behaviour of encapsulated pond snail embryos in diverse natural environments

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SUMMARY

Encapsulated freshwater pond snail embryos display a cilia-driven rotation behaviour that is stimulated by artificially induced hypoxia. Previous studies have suggested that the mixing effect of this behaviour causes enhanced oxygen delivery to embryos within their egg capsules. Despite extensive laboratory-based studies describing this behaviour, it is unclear how this behaviour is used to cope with changes in oxygen concentration and other environmental factors in natural water bodies. We made field measurements of embryo rotation rates in laboratory-reared *Helisoma trivolvis* embryos placed in ponds of different trophic levels that ranged geographically from the southern Alberta prairie to the Rocky Mountains. Abiotic factors including temperature, pH, conductivity and water oxygen concentration were measured to understand how embryonic rotation is influenced by environmental conditions. Results showed that *H. trivolvis* embryos exhibit differences in rotational behaviour depending on the environmental conditions. Temperature and oxygen concentration were the primary factors significantly affecting rotation rates. The effect of oxygen concentration on rotation rates was not as widespread as observed under laboratory conditions, probably because the measured oxygen concentrations were above the range that influences embryonic rotation in the laboratory. The rotational behaviour of laboratory-reared *Lymnaea stagnalis* provided confirmation that embryos of other encapsulated pulmonates exhibit a similar rotational response in natural environments. These results suggest that embryo rotation is influenced by a complex interplay of environmental factors.

Key words: *Helisoma trivolvis*, *Lymnaea stagnalis*, hypoxia, development, behaviour, natural environment.

INTRODUCTION

The pond snail *Helisoma trivolvis* has a broad geographical range within North America, stretching from arctic Canada to Florida and generally thrives in rich, eutrophic environments (Brown, 1991). Adult snails lay egg masses containing encapsulated embryos which undergo direct development into juveniles. Early in development, embryos display a cilia-driven rotational behaviour which is stimulated in response to hypoxia through serotonin release from the embryonic neuron C1s (ENC1s), a bilateral pair of sensory-motor neurons (Diefenbach et al., 1991; Kuang et al., 2002a; Goldberg et al., 2008). Rotational behaviour has been postulated to have originated in planktotrophic larvae of marine gastropods whereby free-swimming veligers utilize bands of cilia for locomotion (Arkett et al., 1987). Consistent with this view is that ENC1 is thought to be a homologue of the para-ampullary neurons of the apical organ, the neural structure that controls locomotion in veliger larvae (Voronezhskaya et al., 1999). Previous laboratory studies have suggested that rotational behaviour is a mechanism to cope with short-term hypoxia by enhancing the rate of oxygen (O_2) diffusion, through mixing of the capsular fluid surrounding the embryo (Hunter and Vogel, 1986; Goldberg et al., 2008).

As a consequence of their wide distribution, *H. trivolvis* are often found in habitats that undergo broad environmental fluctuations. Oxygen levels in freshwater systems are strongly affected by the presence of photosynthetic plants and algae, and productivity is highly influenced by the availability of solar radiation and aquatic nutrients (Modig and Olafsson, 1998). Aquatic temperature fluctuations affect metabolic rates and the solubility of gases such as O_2 and CO_2. In addition, temperature is the primary driver of water circulation in small waterbodies (Chang and Ouyang, 1988; Cole, 1994). Furthermore, pond snail diversity can be affected by the relative abundance of ions (such as Ca^{2+}, Na^{+}, Mg^{2+}, Cl^{-}, NO_3^{-}) within freshwater systems, which are impacted by climate, geography and biotic activity (McKillop and Harrision, 1972; Brown, 1991; Biggs et al., 2005).

Pulmonate gastropod embryos are susceptible to O_2 fluctuations because of their encapsulated state and sedentary nature (Fernandez et al., 2006). As embryos consume O_2, a concentration gradient develops within the egg capsule which, if left undisturbed, can lead to decreased O_2 levels in close proximity to the embryo (Cohen and Strathmann, 1996; Kuang et al., 2002a). In addition to embryonic O_2 consumption, other factors can influence O_2 levels, including fouling organisms on the egg mass (Cohen and Strathmann, 1996; Przeslawski and Benkendorff, 2005) and embryonic behaviour (Hunter and Vogel, 1986; Goldberg et al., 2008). Fouling organisms such as algae, some marine invertebrates, diatoms and protists can settle and attach onto egg masses, which then either consume or produce O_2 (Cohen and Strathmann, 1996; Przeslawski and Benkendorff, 2005). Behaviour of encapsulated embryos also impacts O_2 levels. Rotational behaviour and the underlying ciliary activity stir the capsular fluid, reducing the O_2 gradient and increasing O_2 availability adjacent to the embryo (Strathmann and Chafee, 1984; Goldberg et al., 2008).

Despite the use of *H. trivolvis* as a model organism for neurodevelopment and the neurobiology of embryonic behaviour (Christopher et al., 1996; Christopher et al., 1999; Kuang and Goldberg, 2001; Cole et al., 2002; Kuang et al., 2002a; Kuang et al., 2002b; Doran and Goldberg, 2006; Goldberg et al., 2008), it is

uncertain whether fluctuations in rotational behaviour are due strictly to laboratory-imposed hypoxia or a mix of other environmental factors normally found outside of a controlled laboratory setting. Previously, Goldberg et al. (Goldberg et al., 2008) hypothesized that embryonic rotation is an adaptive behaviour that serves to enhance the rate of O_2 diffusion during periods of hypoxia. We now examine whether pond snail embryos of *H. trivolvis* alter their rotational behaviour in response to environmental fluctuations occurring in natural environments to assess whether this behaviour functions outside of an artificial setting. To accomplish this, O_2 concentration ($[O_2]$), temperature and other environmental factors were measured at five different ponds in Southern Alberta and related to the rotational behaviour displayed by snail embryos at these sites. It was predicted that the rotational behaviour would be most strongly affected by the level of environmental O_2, with the rate of rotation inversely proportional to $[O_2]$. The results suggest that embryonic rotation is controlled through a complex interplay of environmental factors, and not strictly by changes in environmental $[O_2]$.

MATERIALS AND METHODS

Animals

A colony of laboratory-reared *Helisoma trivolvis* Say 1816 and a colony of laboratory-reared Dutch *Lymnaea stagnalis* Linnaeus 1758 were maintained in aquaria containing artificial pond water (APW: 0.025% Instant Ocean, Aquarium Systems, Mentor, OH, USA) and an oyster shell substratum. The aquaria were eumoxic, which was defined as normal $[O_2]$, >4.2 mg O_2 l^{-1} water (Martens et al., 2007). Snails were kept at room temperature (22–23°C) on a 12:12 h light:dark cycle. Their diet consisted of romaine lettuce and trout pellets (NU-WAY: United Feeds, Okotoks, AB, Canada). To facilitate egg laying and egg mass collection, plastic Petri dishes (150 mm diameter) were placed in the aquaria. Egg masses were removed from the collecting dishes with a razor blade and transferred to another Petri dish (70 mm diameter) containing APW for embryonic staging. Embryos of both species were viewed under a dissection microscope and staged as a percentage of intracapsular development, with stage E0 corresponding to the zygote at 0% development and stage E100 corresponding to hatching at 100% development (Goldberg and Kater, 1989; Diefenbach et al., 1998; Voronezhskaya et al., 1999). All embryos used in this study were of embryonic stage E20–E30 for *H. trivolvis* and E35–E40 for *L. stagnalis*.

The source population of our *H. trivolvis* was originally from Oregon and bred initially by Dr Stanley Kater in the 1970s (Kater, 1974). This population of *H. trivolvis* probably lived in freshwater streams, ponds and lakes similar to where wild *H. trivolvis* populations are now found. A founder effect is possible, but it is likely that additional wild *H. trivolvis* individuals or snails bred from different source populations have been added over the decades, which may mitigate a founder effect. However, there is no reason to think that these animals would be substantially different from other model organisms when compared with their wild counterparts. Their generation time is approximately 5 weeks (Kater, 1974); over 35 years that adds up to roughly 350 generations, which is probably not long enough to significantly alter behavioural or physiological responses in these gastropods (Brown, 1991; Diefenbach, 1990).

We limited the experiments to laboratory-raised snails since the main goals were to test (1) whether experimentally induced changes in embryonic rotation observed under highly controlled laboratory conditions reflect environmentally induced changes in natural environments, and (2) whether O_2 or other environmental variables are the primary regulators of rotational activity. By using a single laboratory-reared strain in this initial study, the confounding effects of strain differences between isolated populations are mitigated (Orr et al., 2009).

Embryonic rotation rate measurements

Embryonic rotation was recorded using an imaging system composed of a dissection microscope (StemiSR, Zeiss, Toronto, ON, Canada) mounted with a digital video camera (Qicam Fast, cooled color 12-bit, Qimaging, Surrey, BC, Canada). Movies of embryonic behaviour were captured and analyzed using the time-lapse function of Northern Eclipse imaging software (Northern Eclipse 6.0, Empix Inc., Mississauga, ON, Canada). Rotational behaviour was recorded at 0.25 frames s^{-1} by the time-lapse function and played back at 180 times the recording speed to facilitate the counting of rotations. The behaviour was sufficiently slow (normally 0.7±0.1 r.p.m.) to preclude the possibility of aliasing artifacts. The average rate of rotation was analyzed by counting the number of rotations during a 5–10 min video; counts were accurate to within half a rotation.

Delivery of controlled levels of O_2 in the laboratory

To examine rotational responses to $[O_2]$, egg masses were exposed to various O_2 levels ranging from eumoxia to anoxia. For hypoxia experiments, calibrated ratios of O_2 and N_2 were mixed using mass flow controllers (Smart Trak, digital mass flow, Sierra Instruments, Monterey, CA, USA) driven by Chinook Scientific software (version 2.0; written by R. J. Wilson, University of Calgary, Calgary, AB, Canada) and verified using an analyzer (Model 3750, Illinois Instruments, Johnsburg, IL, USA). Gas mixtures were bubbled into a Petri dish (150 mm×25 mm) which was sealed with Parafilm and tilted approximately 10 deg to facilitate the flow of gas through the dish. The egg mass was stabilized with dental wax at the lower edge of the dish. Gas mixtures were continually introduced (1.8 l min^{-1}) during the experiments through fluorinated ethylene propylene tubing (Cole Parmer, Montreal, PQ, Canada) and exhausted *via* a small opening at the elevated side of the dish.

To construct a concentration–response curve of hypoxia-stimulated embryonic rotation, different levels of O_2 were bubbled into a sealed Petri dish as described above. Rotation measurements were taken 10 min after the start of gas perfusion. The $[O_2]$ used were 7.9, 3.3, 2.9, 2.5, 2.1, 1.7, 1.2, 0.8, 0.5 and 0.0 mg O_2 l^{-1} water.

Control of pond water temperature in the laboratory

The effect of temperature upon embryonic rotational behaviour was examined using a circulating water bath (Lauda K-2/RD, Brinkmann Instruments, Mississauga, ON, Canada) that pumped cooled or heated fluid through Tygon tubing that was placed in a Petri dish containing a stabilized egg mass. Measurements of embryonic rotation were taken initially at room temperature, then at either 5, 10, 15, 20, 25, 30, or 35°C at 10 min intervals over 1 h. Following the temperature treatments, the APW was allowed to return to room temperature and rotation was again recorded at 10 min intervals. The water temperature was continuously monitored adjacent to the egg mass during the experiments, using a glass thermometer (VWR International, Edmonton, AB, Canada).

Field measurements

To examine the impact of various environmental factors on embryonic rotational behaviour in natural settings, five different ponds around southern Alberta were chosen: Upper Lloyd, Belly River Drainage, Lloyd Lower, Sibbald Flats and Rosebud Complex. Refer to Table 1 for further detail regarding the field sites. Five environmental variables were monitored at each pond, including

Table 1. Names and locations of ponds used for field studies

Pond	Trophic level	Water source	Location	Coordinates
Upper Lloyd	Oligotrophic	Spring	Bragg Creek, AB, Canada	50° 56′ 46″ N, 114° 38′ 55″ W
Lower Lloyd	Moderately eutrophic	Upper Lloyd	Bragg Creek, AB, Canada	50° 56′ 47″ N, 114° 38′ 55″ W
Rosebud Complex	Highly eutrophic	Cow pasture run-off	*ca.* 50 km NE of Calgary, AB, Canada	51° 17′ 28″ N, 113° 27′ 04″ W
Sibbald Flats	Moderately eutrophic	Creek	Junction of Hwy 68 and Powderface Tr., AB, Canada	51° 01′ 25″ N, 114° 51′ 33″ W
Belly River Drainage	Oligotrophic	Spring	*ca.* 30 km south of Fort Macleod, AB, Canada	49° 31′ 36″ N, 113° 16′ 36″ W

the amount of dissolved O_2 in pond water (oxygen meter, YSI model 57; Yellow Springs, OH, USA), pond water conductivity (EC/TDS, Hanna Instruments, Laval, PQ, Canada), pond water pH (colorpHast pH-indicator strips, EMD Chemicals Inc., Gibbstown, NJ, USA) and water and air temperature (EC/TDS, Hanna Instruments).

Egg masses of both species were brought from the laboratory and were placed in plastic containers which had insect screen (1 mm mesh) on the top and bottom to allow for ample water circulation and interaction with the surroundings while ensuring that the egg masses were contained. Pond snails primarily lay their egg masses on floating or attached vegetation close to the shore and near the surface of the water (R.B.S., unpublished observations), therefore the containers were placed approximately 1 m from the shore and the buoyancy of the plastic held the containers near the water surface. Egg masses were acclimated to the pond environment for at least 1 h prior to commencing the recording of rotational behaviour.

For measurements of rotation rates, egg masses were transferred into jars containing pond water and carried over to the microscope, which was typically 5–25 m away from the pond. The egg masses were removed from the jars and placed into Petri dishes containing pond water and rotation was measured. Once rotation was measured, egg masses were placed back into the plastic containers and returned to the pond. All environmental measurements were taken at the location in the pond of the egg masses, and the same site in each pond was used upon repeated visits.

Measurement of rotational behaviour was conducted using the same set-up as described above, although rotation rates were measured over a 7-min period in order to minimize time out of the pond. To verify that conditions were similar throughout the recording period, the temperature of the pond water in the Petri dish was measured at the start and finish of the recordings, and compared with the actual pond water temperature. The water temperature in the Petri dish was 1.3±0.2°C higher at the start and 3.3±0.3°C higher at the finish than the pond water temperature. There was no consistent change in the pattern of rotation dynamics from the start to the finish of each 7 min recording period (data not shown).

Rotations were measured at various time points throughout the day at 1-h intervals during daylight hours over a period of 3 months from June to August 2008 and during August 2009. Measurements at the Belly River drainage were performed during August 2007 and were made at approximately 4-h intervals during daylight hours.

Data analysis

Results were expressed as means ± standard error (s.e.m.). Tests were performed for the effects of $[O_2]$, water temperature and ponds on embryonic rotation rate data using a one-way ANOVA followed by a *post-hoc* Tukey's test. A general linear model was constructed to determine the significance, if any, of environmental variables on rotation rates. Statistical tests were performed using the statistical analysis programs GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA) and SYSTAT 10.2.01 (SYSTAT Software, Inc., Chicago, IL, USA).

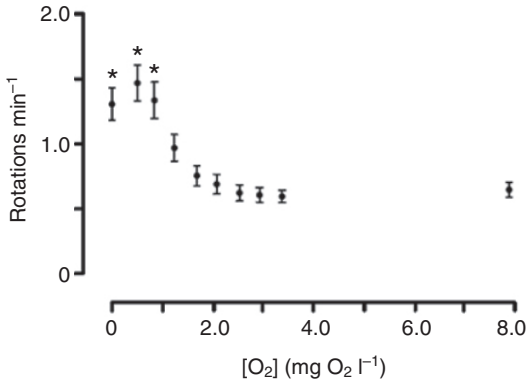

Fig. 1. Embryo rotation rate in hypoxia is $[O_2]$ dependent under laboratory conditions. Embryo rotation increased in a dose-dependent manner with decreases in $[O_2]$ (measured after 20 min of exposure to experimental $[O_2]$; N=15). *P<0.001 (ANOVA followed by Tukey's test) as compared with an $[O_2]$ of 7.9 mg O_2 l^{-1} water.

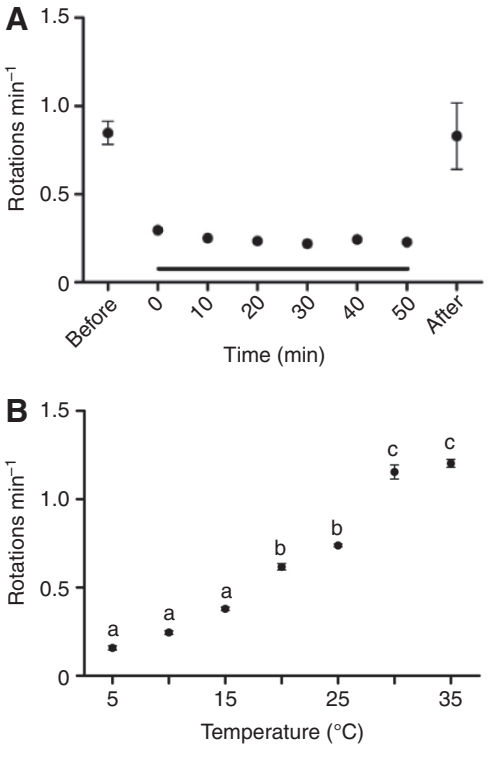

Fig. 2. Rotation response to temperature under laboratory conditions. (A) Rotation rates were measured at room temperature (~22±1°C), during a 60 min exposure to 10°C and after returning to room temperature; black line indicates the duration of the experimental temperature. Each point represents the average rotation rate in the 10 min following the time shown. (B) Rotational behaviour in response to various temperatures. Different letters indicate significance differences between treatments: P<0.001 (N=20).

For statistical purposes, each embryo was considered an '*N*' and embryos from at least two different egg masses were used unless otherwise noted. Although it would have been preferable to include data from larger numbers of egg masses, with each egg mass representing a sample measurement, this approach creates logistical challenges that would significantly slow the collection of sufficient data sets. Our studies of rotational behaviour over two decades have consistently revealed a paucity of variation between egg masses and thus form the basis of our current statistical approach.

RESULTS

Embryonic rotation rate is [O_2] dependent in the laboratory

As a foundation for field experiments on the relative role of O_2, we examined the rotation rate of *H. trivolvis* embryos exposed to a range of [O_2] in the laboratory. The rate of embryonic rotation at atmospheric O_2 levels (7.9 mg O_2 l^{-1} water) was 0.65±0.06 r.p.m. (*N*=15) and did not significantly change until the [O_2] was reduced to 0.8 mg O_2 l^{-1} water ($F_{9,137}$=13.13, P<0.0001). As hypoxia became more pronounced, the rotation rate increased and peaked at 0.5 mg O_2 l^{-1} water, where the rotation rate was about 2.3 times the basal rate (1.47±0.14 r.p.m., *N*=3; Fig. 1). Similar to other studies (Kuang et al., 2002a), embryonic rotation was maximal at 0.5 mg O_2 l^{-1} water and reached half-maximal rotation at 1.2 mg O_2 l^{-1} water.

Temperature exerts a strong effect on rotation rate

As temperature is an environmental variable capable of altering behavioural and physiological functions in other animal systems, we first examined the rotational response to temperature within controlled laboratory settings. The response to temperature was rapid as embryos altered their rotation rate within 10 min and maintained it at that level for the duration of the experiment (Fig. 2A). This change was reversible, as embryonic rotation recovered to pre-treatment levels in all cases following a return to room temperature (22°C). For example, exposure to 10°C resulted in approximately a 50% drop in rotation rate within 10 min, and there were no further changes until embryos were returned to room temperature (Fig. 2A).

Subjecting embryos to temperatures ranging from 5–35°C resulted in significant changes to embryonic rotation rates ($F_{6,147}$=42.4, P<0.0001; Fig. 2B). The lowest mean rotation rate was observed at 5°C (0.16±0.01 r.p.m., *N*=20) and the highest was at 35°C (1.20±0.02 r.p.m.; *N*=20). The difference between these two points represents an approximate sevenfold increase (Fig. 2B).

Embryo rotational behaviour is significantly altered by environmental fluctuations in natural ponds

Fluctuations of water and air temperature, [O_2], pH and conductivity occurred at all five ponds. Embryonic rotation rates also varied at each pond location so we attempted to determine the environmental source of the behavioural variation.

The Upper Lloyd pond was an oligotrophic aquatic environment that experienced moderate changes in both water temperature and O_2 during the day. Concentrations of O_2 remained high throughout the season observed, averaging around 10 mg O_2 l^{-1} water with minimal daily fluctuations (Fig. 3A). Similarly, water temperature, pH and conductivity remained stable between measurement periods. Water temperature varied by 2 to 4°C, and pH and conductivity were 7.6±0.1 units and 437±3.3 μS cm^{-1}, respectively. Rotation rates of *H. trivolvis* embryos averaged 0.6±0.1 r.p.m. with little intraday variation (Fig. 3B), and were significantly affected by both pond pH and water temperature (Table 2).

The Lower Lloyd pond was moderately eutrophic and underwent moderate variations in both water temperature and O_2. Intraday changes were observed for water temperature, with temperature typically lowest in the mornings and highest in the early afternoon. By contrast, O_2 levels changed little during the day. Neither pH

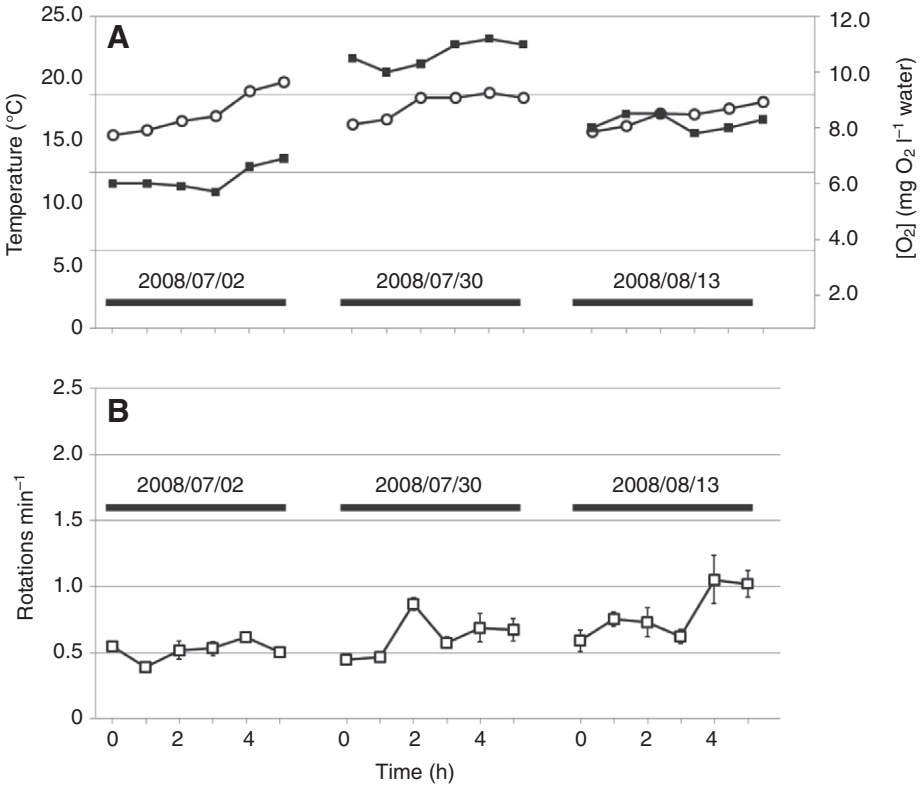

Fig. 3. Measurements of water temperature, [O_2] and rotation rates at Upper Lloyd Pond during July and August 2008. (A) Measurements of water temperature (°C; open circle symbols) and [O_2] (mg O_2 l^{-1} water; square symbols). (B) Embryo rotation rate of *H. trivolvis* (*N*=10). All measurements were taken 1 h apart, starting at 09:00 h (0 h).

Table 2. Results of a general linear model analysis testing the effect of various environmental factors (water temperature, air temperature, oxygen, pH and conductivity) on rotation rates of *H. trivolvis* embryos

Upper Lloyd Pond					
Dependent variable: r.p.m.	*N*=149	Multiple R: 0.359		Squared multiple R: 0.129	
Adjusted squared multiple R: 0.117			Standard error of estimate: 0.286		
Effect	Coefficient	s.e.	Tolerance	*t*	*P* (2 tail)
Constant	–3.199	0.833	–	–3.840	0.000
Water temperature	0.069	0.026	1.000	2.677	0.008
pH	0.336	0.089	1.000	3.785	0.000
Analysis of variance					
Source	SS	d.f.	Mean square	*F*-ratio	*P*
Regression	1.769	2	0.884	10.776	0.000
Residual	11.980	146	0.082		
Lloyd Lower Pond					
Dependent variable: r.p.m.	*N*=243	Multiple R: 0.187		Squared multiple R: 0.035	
Adjusted squared multiple R: 0.023			Standard error of estimate: 0.435		
Effect	Coefficient	s.e.	Tolerance	*t*	*P*(2 tail)
Constant	–1.495	1.039	–	–1.440	0.151
pH	0.295	0.146	0.961	2.023	0.044
Air temperature	0.027	0.011	0.963	2.353	0.019
Oxygen	0.003	0.014	0.957	0.223	0.824
Analysis of variance					
Source	SS	d.f.	Mean square	*F*-ratio	*P*
Regression	1.628	3	0.534	2.872	0.037
Residual	45.163	239	0.189		
Rosebud Complex Pond					
Dependent variable: r.p.m.	*N*=221	Multiple R: 0.224		Squared multiple R: 0.050	
Adjusted squared multiple R: 0.041			Standard error of estimate: 0.696		
Effect	Coefficient	s.e.	Tolerance	*t*	*P* (2 tail)
Constant	–0.176	0.340	—	–0.518	0.605
Conductivity	0.000	0.000	0.905	2.211	0.028
Water temperature	0.024	0.013	0.905	1.767	0.079
Analysis of variance					
Source	SS	d.f.	Mean square	*F*-ratio	*P*
Regression	5.571	2	2.786	5.753	0.004
Residual	105.546	218	0.484		

d.f., degrees of freedom; s.e., standard error; SS, sum of squares.

(6.5±0.1) nor conductivity (313±6.6 $\mu S\,cm^{-1}$) underwent obvious daily changes, but over the summer conductivity and pH gradually increased. Rotation rates of *H. trivolvis* embryos did not display a typical pattern throughout the day and appeared independent of both water temperature and O_2 (Fig. 4B). By contrast, they were significantly affected by both pond pH and air temperature (Table 2).

The Rosebud pond was highly eutrophic, largely due to the high levels of bovine faecal matter contained in the run-off water that fed the pond. On two of the three days, the $[O_2]$ increased over 500% from lows of approximately 1.5 $mg\,O_2\,l^{-1}$ water to over 9.5 $mg\,O_2\,l^{-1}$ water. Likewise, water temperature also underwent large increases of about 10°C each day (Fig. 5A). Conductivity was very high in this pond (2247±80.4 $\mu S\,cm^{-1}$), approximately three times greater than the other ponds. The pH was also higher (8.3±0.3), especially on the last sampling day when it was moderately alkaline at a pH value of 8.7. The rotation rate of *H. trivolvis* embryos showed some intraday large fluctuations (Fig. 5B), and was significantly affected by conductivity (Table 2).

The two other ponds examined, Sibbald Flats and Belly River Drainage, also exhibited fluctuations in water temperature and $[O_2]$ with negligible changes in pH and conductivity. Embryo rotation rates varied as well, and rotation rates were found to be significantly affected by $[O_2]$ (data not shown). In both locations, rotation rates and $[O_2]$ were positively correlated.

Rotation rates of *H. trivolvis* embryos adjust to the pond environment

Examining aggregated rotation rates of *H. trivolvis* embryos at each pond suggested that embryos alter their rotational activity to occupy a select range depending upon the characteristics of the pond. In oligotrophic ponds, such as Upper Lloyd and Belly River ponds, embryo rotation rates were slower overall , within a fairly narrow range (mean rotation rate 0.6±0.1 and 0.8±0.1 r.p.m., respectively), whereas in the highly eutrophic Rosebud pond the embryos exhibited a large range of rotation rates (mean rotation rate 1.0±0.1 r.p.m.). In moderately eutrophic environments such as Lower Lloyd and Sibbald ponds embryo rotation rates were more intermediate (mean rotation rate 1.1±0.1 and 1.2±0.1 r.p.m., respectively) (Fig. 6). Rotation rates were significantly different between some of the ponds sampled, indicative of the different conditions occurring at each pond ($F_{4,103}$=6.4119, P<0.0001).

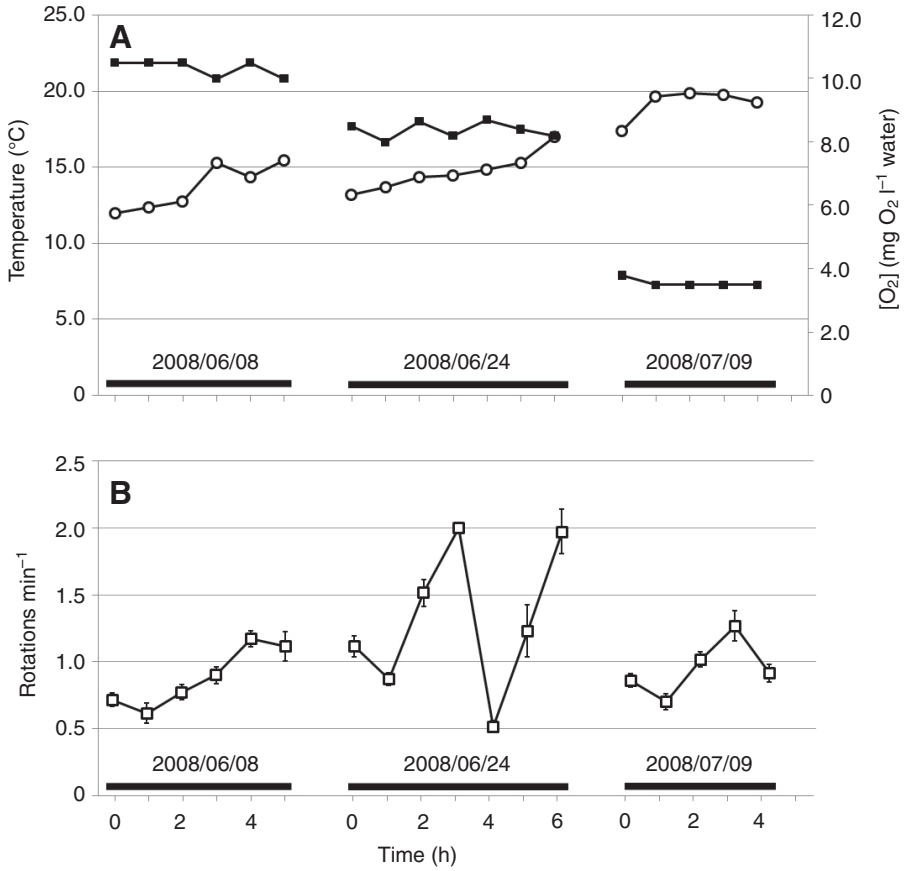

Fig. 4. Measurements of water temperature, $[O_2]$ and rotation rates at Lower Lloyd Pond during June and July 2008. (A) Measurements of water temperature (°C; open circles) and $[O_2]$ (mg O_2 l^{-1} water; square symbols). (B) Embryo rotation rates of *H. trivolvis* (N=5–10). All measurements were taken 1 h apart, starting at 09:00 h (0 h).

Rotational response of laboratory-reared *Lymnaea* embryos

As other pulmonate gastropod embryos undergo rotational behaviour similar to *H. trivolvis* (Goldberg et al., 2008), we compared the rotational behaviour of laboratory-reared *Lymnaea stagnalis* embryos with laboratory-reared *H. trivolvis* embryos at the Lower Lloyd pond. On the days tested, little variability was observed in any of the environmental measurements taken, suggesting relatively stable conditions. Likewise, the behaviour of both species was quite stable. Rotation rates of *L. stagnalis* were significantly higher than those of *H. trivolvis* ($F_{1,126}$=115.90, P<0.0001), however rotation rates of both species were similar to their respective basal rotation rates measured in the laboratory (Fig. 1) (Goldberg et al., 2008). Rotation rates of both species did not change significantly throughout the course of the day ($F_{6,126}$=1.14, P>0.05), probably because of the minimal environmental variation.

DISCUSSION

From our 'stir-bar hypothesis' (Goldberg et al., 2008) and prior results (Kuang et al., 2002a) (Fig. 1), we had predicted that rotation rates would be inversely proportional to environmental $[O_2]$. *H. trivolvis* embryos, which normally rotate at stable rates under laboratory conditions, changed their rotation behaviour in various ponds, but this could not be directly explained by the environmental variables measured. Our data suggested that although O_2 levels are an important determinant of rotational behaviour in the laboratory, there are other environmental variables which strongly influence rotation rates in natural pond settings.

Analysis of specific environmental factors affecting rotation rates indicated that temperature at three ponds played an important role in altering rotation rates. Water temperature significantly affected rotation in Upper Lloyd pond and had a strong but not significant effect in the Rosebud pond, whereas air temperature had a significant effect at Lower Lloyd pond (Table 2). At Lower Lloyd pond, air temperature may have had a significant effect rather than water temperature because of effects related to solar radiation such as UV (Przeslawski et al., 2004; Wahl, 2008). Temperature is believed to have an effect on embryonic rotation because of the tight relationship between metabolism and temperature in poikilotherms. Gastropod development is temperature dependent, as metabolic rates increase at higher temperatures and decrease at lower temperatures (Harris and Charleston, 1977; Moran and Woods, 2007). Embryo rotation is an energy intensive activity that is probably responsive to changes in metabolic activity, because of both the high demand on ATP production and the extensive biomechanical and energetic processes underlying ciliary beating (Goldberg et al., 2008). Although outside the scope of the current study, it would valuable to measure metabolic rate in both laboratory and field settings to gain a better understanding of the metabolic costs of embryonic rotation under various environmental conditions.

It is surprising that O_2 only weakly affected embryonic rotation in natural ponds because O_2 fluctuations pose a challenge for encapsulated embryos (Strathmann and Strathmann, 1995; Fernandez et al., 2006). Oxygen levels change daily and seasonally because of the presence of photosynthetic organisms and fouling organisms that increase or decrease O_2 levels, respectively, immediately surrounding the egg mass (Chang and Ouyang, 1988; Cole, 1994; Cohen and Strathmann, 1996; Przeslawski and Benendorff, 2005). Additionally, the water solubility of O_2 is temperature dependent (Cole, 1994); however, the drop in solubility due to the rise in temperature was not enough to affect $[O_2]$ in the studied ponds because of the constant addition of O_2 from

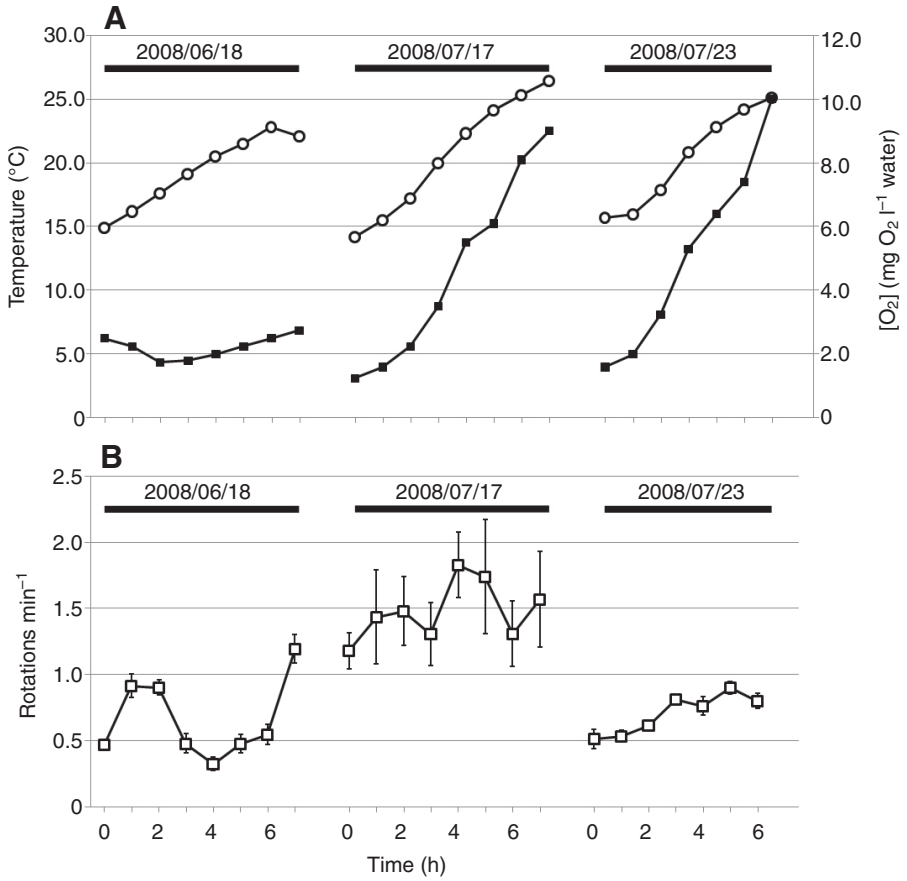

Fig. 5. Measurements of water temperature, $[O_2]$ and rotation rates at Rosebud Complex Pond during June and July 2008. (A) Measurements of water temperature (°C; open circles) and $[O_2]$ ($mg\,O_2\,l^{-1}$ water; square symbol). (B) Embryo rotation rate of *H. trivolvis* (*N*=10). All measurements were taken 1 h apart, starting at 09:00 h (0 h).

photosynthesis. A 10°C increase in water temperature, which was among the largest increases observed in the ponds, can decrease the concentration of O_2 by approximately $2\,mg\,O_2\,l^{-1}$ water (Cole, 1994). However, O_2 reductions due to temperature and consumption can easily be offset by the production of O_2 by photosynthesis, as some species of algae can produce over $40\,mg\,O_2\,g^{-1}$ tissue h^{-1}, which may also explain why some ponds become supersaturated with O_2 (King and Schramm, 1976).

That $[O_2]$ did not have as large an effect on rotation rates as was originally predicted may have been due to the range of O_2 levels that were reached. In laboratory experiments, the maximal rotational response occurred at $0.5\,mg\,O_2\,l^{-1}$ water (Fig. 1). The concentration of O_2 in the ponds never reached this low level, nor did they reach the level where half-maximal rotation occurs at $1.1\,mg\,O_2\,l^{-1}$ water; the lowest recorded $[O_2]$ was $1.3\,mg\,O_2\,l^{-1}$ water. Generally, the water was not hypoxic enough to evoke anything more than a mild stimulation of the rotational response (Kuang et al., 2002a) (Fig. 1). The absence of severe hypoxia in pond environments is probably a factor in why rotation rates were not strongly affected by O_2 levels, as simply, ponds almost never became hypoxic enough to warrant such a behavioural response. Perhaps measurements of O_2 further from the surface where some egg masses are deposited would reveal lower O_2 levels in the range that would strongly influence embryonic rotation. Similarly, eutrophic ponds probably became depleted of O_2 during the night, and a full circadian analysis of rotational behaviour may reveal the expected link between O_2 and rotation. The five ponds examined in this study spanned the range of oligotrophic to eutrophic conditions, and revealed a trend of increased rotational variation associated with eutrophy. However, a systematic analysis of multiple ponds at each of the three trophic levels is required to validate this interesting tendency.

As only five environmental factors were measured, it was difficult to gain a complete appreciation for the breadth of influences that may affect embryonic rotational behaviour. Other environmental factors which are known to affect gastropods include solar radiation (including ultra-violet radiation) and ions such as Ca^{2+} (McKillop and Harrison, 1972; Przeslawski et al., 2004; Wahl, 2008). The location of planorbid egg mass deposition has been observed to be influenced by the ability of the eggs to survive exposure to ultra-

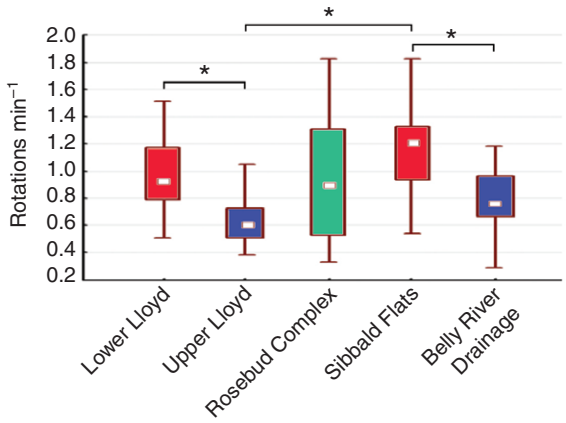

Fig. 6. Range of rotation rates of *H. trivolvis* embryos in each of the ponds (shown as min–max box and whisker plot). *H. trivolvis* rotation rates were significantly different between the ponds sampled ($F_{4,103}$=6.4119, P<0.0001). The Tukey's *post-hoc* test revealed significant differences in rotation rates between Upper Lloyd and Lower Lloyd, Sibbald Flats and Upper Lloyd, and Sibbald Flats and Belly River Drainage ponds (*: P<0.05). The different colours represent approximate trophic levels: blue, oligotrophic; red, moderately eutrophic; green, highly eutrophic.

violet radiation (Przeslawski, 2004; Przeslawski et al., 2004; Wahl, 2008). Ion concentration may affect embryo development as the egg capsule surrounding the embryo has a positive osmotic pressure (Taylor, 1973), therefore highly saline environments may cause the capsular membrane to shrivel. Additionally, certain ions such as Ca^{2+} are important for development of embryonic structures. Pulmonate species found in Ca^{2+}-poor environments suffer from slower growth and have thinner shells than those in Ca^{2+}-rich environments (McKillop and Harrison, 1972). Ion concentration probably has an effect on embryonic rotational behaviour, as pond conductivity and pH were found to have significant effects on rotation rates in three ponds (Table 2). Previous laboratory studies found that both pH and conductivity within a normal healthy environmental range have very little effect on rotational behaviour (Kuang, 2002). This warrants further examination, especially under controlled laboratory conditions. Furthermore, it is conceivable that additional environmental factors, both abiotic and biotic, may influence embryonic rotation.

Embryos of the pulmonate gastropods, *L. stagnalis* and *H. trivolvis*, share common characteristics, including cilia-driven rotational behaviour within egg capsules and responsiveness to hypoxia (Goldberg et al., 2008; Byrne et al., 2009). The rotational behaviour of these species was compared in natural environments to assess whether they employed similar ventilation strategies. On both days tested, the temperature and O_2 levels were extremely stable, which correlated well to relatively stable rotation rates. However, these rates differed significantly between the species, probably a consequence of evolutionary differences, which include differential patterning of ciliary bands and different neurotransmitter phenotypes present (Diefenbach et al., 1991; Goldberg et al., 1994; Voronezhskaya et al., 1999). Collectively, these experiments suggest that rotational behaviour serves a similar function in both species.

Our data demonstrate that embryo rotation in *H. trivolvis* is a dynamic behaviour that varies depending on a complex interplay of multiple factors. Temperature and O_2 are believed to act as primary drivers of this behaviour, as supported by laboratory results; however, other environmental factors have large influences over this behaviour. Progress on this question would probably require the development of a mathematical model that assesses the relative roles of additional environmental factors. Additionally, a beneficial next step would be to compare multiple wild populations of *H. trivolvis* and *L. stagnalis* to elucidate the consequences of extensive inbreeding and geographical isolation on embryo rotation.

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