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RESEARCH ARTICLE

Development of cardiovascular function in the marine gastropod *Littorina obtusata* (Linnaeus)

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SUMMARY

The molluscan cardiovascular system typically incorporates a transient extracardiac structure, the larval heart, early in development, but the functional importance of this structure is unclear. We documented the ontogeny and regulatory ability of the larval heart in relation to two other circulatory structures, the true heart and the velum, in the intertidal gastropod *Littorina obtusata*. There was a mismatch between the appearance of the larval heart and the velum. Velar lobes appeared early in development (day 4), but the larval heart did not begin beating until day 13. The beating of the larval heart reached a maximum on day 17 and then decreased until the structure itself disappeared (day 24). The true heart began to beat on day 17. Its rate of beating increased as that of the larval heart decreased, possibly suggesting a gradual shift from a larval heart-driven to a true heart-driven circulation. The true heart was not sensitive to acutely declining P_{O_2} shortly after it began to beat, but increased in activity in response to acutely declining P_{O_2} by day 21. Larval heart responses were similar to those of the true heart, with early insensitivity to declining P_{O_2} (day 13) followed by a response by day 15. Increased velum-driven rotational activity under acutely declining P_{O_2} was greatest in early developmental stages. Together, these findings point to cardiovascular function in *L. obtusata* larvae being the result of a complex interaction between velum, larval and true heart activities, with the functions of the three structures coinciding but their relative importance changing throughout larval development.

Key words: invertebrate circulation, cardiac ontogeny, extracardiac circulation, transitory structures, velum, larval heart, direct development, Mollusca, ecophysiology, hypoxia.

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INTRODUCTION

Most of our knowledge of invertebrate cardiovascular physiology stems from adult organisms (Maynard, 1961; McMahon et al., 1997a; McMahon et al., 1997b). Yet, there is growing evidence that the development of the cardiovascular system in many groups is a complex and dynamic process, often involving phases that function in a very different way to one another and to the adult system (McMahon et al., 1997a; McMahon et al., 1997b; Reiber and Harper, 2001; Spicer, 2001; McMahon et al., 2002; Harper and Reiber, 2004). In insects, for example, there are distinct patterns of activity of the dorsal abdominal vesicle associated with larval, pupal and adult stages (Smits et al., 2000), whereas, for crustaceans, an ontogenetic shift has been suggested to occur from myogenic to neurogenic control of heart, although some groups appear to retain a myogenic control into adulthood (e.g. Spicer, 2001).

As well as such shifts associated with the development of a single cardiovascular organ, several invertebrate groups can appropriate alternative means of circulation during early development. In some cases this is through the 'co-option' of structures used for other functions later in development. For example, a circulatory role has been suggested for the gut in early-stage crustacean embryos (McMahon et al., 1997b), a role that was confirmed experimentally in brine shrimp (Spicer, 2006). At the same time, some invertebrate groups possess transient structures that play a role in circulation. Early cardiovascular development in many molluscs, for example, is marked by the appearance of an extracardiac structure termed the 'larval heart' (Salensky, 1872; Franc, 1940; Kohn, 1961; D'Asaro, 1965; Switzer-Dunlap and Hadfield, 1977; Little et al., 1985; Collin and Wise, 1997; Gibson, 2003; Gittenberger, 2003). The 'true' heart develops later and, in most species, there is a transitional phase during which the two hearts operate concomitantly before the larval heart degenerates and disappears (Delsman, 1914; Kriegstein, 1977). Despite the repeated identification of the larval heart in developing molluscs, little is known about its functional role.

Werner (Werner, 1955) provided one of the most detailed morphological descriptions of the larval heart for the slipper limpet *Crepidula fornicata*. He described it as a thin-walled, non-ciliated ectodermal vesicle, the front opening of which connected to the velum and the foot, and the rear to the intestine; unidirectional movement of body fluid was observed from front to back. The velum is a transitory, ciliated structure that has a role in larval feeding and locomotion (Fretter and Graham, 1962; Fretter, 1967; Chaparro et al., 2002b; Chaparro et al., 2002a), and gas exchange (Fioroni, 1966). Werner concluded that the primary task of the larval heart was to circulate oxygenated haemolymph from the velum into the intestine and that, by pumping fluid from the velum and foot into the intestine, it could also drive contraction of the velum (Werner, 1955). Based on Werner's observations, we might predict that the functional ontogenies of the larval heart and velum, both transitory larval structures, should coincide, with larval heart function decreasing and then ceasing alongside the disappearance of the velum, and the

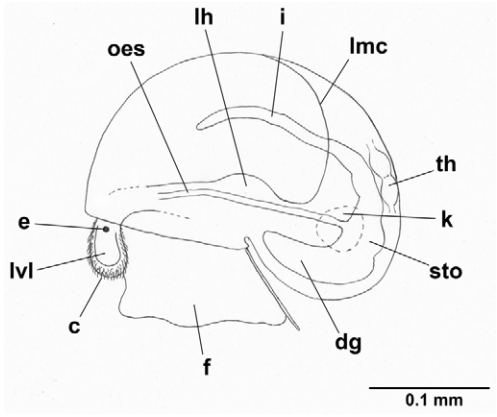

Fig. 1. Diagrammatic representation of a 17 day old *Littorina obtusata* larva, seen from the left side. Both larval (lh) and true hearts (th) are present. c, cilia; dg, digestive gland; e, eye; f, foot; i, intestine; k, kidney; lmc, limit of mantle cavity; lvl, left velar lobe; oes, oesophagus; and s, stomach.

true heart taking over as the main driver of cardiovascular circulation.

Consequently, the main aim of this study was to investigate the development of cardiovascular function in the flat periwinkle, *Littorina obtusata* (Fig. 1). This intertidal gastropod is amenable to experimental manipulation and observation as it undergoes direct encapsulated development within a gelatinous egg mass, hatching (after ~30–35 days at 15°C) as a crawling juvenile. Whilst many gastropod egg capsules are considered to be freely permeable to water (e.g. Galtsoff et al., 1937; Carriker, 1955; Pechenik, 1983), the jelly matrix enclosing the embryos has been found to constrain oxygen diffusion into the egg mass (Brante et al., 2009). Furthermore, embryos are often crowded within egg masses and are frequently exposed to large fluctuations in O_2 partial pressure (P_{O_2}) in the intertidal zone (Raffaelli and Hawkins, 1996). Encapsulated embryos of *L. obtusata* are therefore likely to be exposed to reduced P_{O_2}, and so O_2 supply may be one of the main challenges for this species during its early development, making it an interesting model for investigating the ontogeny of cardiovascular function.

Firstly, we document the patterns of appearance (and where appropriate disappearance) of the larval and true hearts and the velum during larval development, superimposing larval heart and true heart activity at different stages of development. We also incorporate a measure of rotational behaviour during larval development as this behaviour is thought to play a role in O_2 uptake by larvae, providing circulation by mixing fluids within the egg capsule (Goldberg et al., 2008; Byrne et al., 2009). We used information of these patterns to test the hypothesis that larval heart function is intimately related to the production of a functional velum and that the development of these structures would coincide, with a subsequent reduction in larval heart activity as the velum degenerates and as circulatory function is assumed by the true heart. Secondly, we investigated how aspects of the regulatory ability of both the larval heart and true heart change through ontogeny, by measuring how the effects of acutely declining P_{O_2} on both structures change through development.

MATERIALS AND METHODS

Animal material

Adult *L. obtusata* (N=90) were collected by hand from the low intertidal zone at Mount Batten, Plymouth, Devon, UK (50°21.34′N, 04°29.78′W) during February 2010. Animals were transported to the laboratory in buckets containing the weed they were collected on (*Ascophyllum nodosum*). In the laboratory, they were maintained at 15±0.5°C in Plexiglas aquaria (volume 6 l, 15 individuals per aquarium) containing filtered, aerated seawater [salinity (S)=34±1] and fed *ad libitium* with *A. nodosum*. Seawater was changed weekly. Egg masses were subsequently laid on algal fronds. The egg masses were carefully removed, using forceps, within 24 h of being deposited, and then divided into smaller egg batches (~10 embryos) using a razor blade. Batches were then transferred to multiwell plates (24 wells, Nunclon Multidish, Fisher Scientific UK, Loughborough, Leics, UK). Each well contained one egg batch in 3 ml of seawater and was maintained at 15±0.5°C. Seawater in each well was replaced daily (90%).

Observation chamber

Observations on larval and true heart activity (expressed as beats min^{-1}) and rotational behaviour of larvae (% time spent rotating) for individuals from each replicate egg batch were made in a transparent, flow-through chamber (5×2.5×1 cm) six times during development over a period covering the ontogeny of both larval and true hearts (i.e. 13, 15, 17, 19, 21 and 22 days post-laying). Egg batches were fixed in position in the flow-through chamber using a small amount of Vaseline petroleum jelly (Unilever, London, UK). The larvae were introduced into the flow-through chamber 10 min prior to the start of the experiments. The chamber was supplied with filtered natural seawater (S=33.5±0.2, pH 8.04±0.03), pumped using a peristaltic pump (DC motor, flow rate 6.2 ml min^{-1}; Maxon Motor, Finchampstead, Berks, UK) from a reservoir (volume 5 l) to the flow-through chamber, *via* an O_2 electrode (Strathkelvin Instruments, North Lanarkshire, UK) housed in a microcell (MC100 Microcell, Strathkelvin Instruments). The O_2 electrode was coupled to an O_2 meter (Model 781, Strathkelvin Instruments). Water in the reservoir was replaced daily. Water in the flow-through chamber was maintained at a temperature of 15.3±0.2°C using an electronic water bath (Grant LTC 6-30, Grant Instruments**,** Cambridge, UK).

Effect of acutely declining O_2

Control egg batches were exposed in the flow-through chamber to normoxic sea water (>90%) for 1 h. Reservoir seawater was aspirated with compressed air after scrubbing (3 mol l^{-1} KOH) to remove CO_2. Hypoxia treatments were constructed by aspirating reservoir seawater with compressed N_2 gas such that the P_{O_2} of the water decreased at a rate of ~1.5% min^{-1} [a dissolved O_2 saturation of 100% is equivalent to 8.565 mg l^{-1} (Weiss, 1970)]. A lid constructed from Styrofoam was placed in the reservoir to reduce the air–water interface and so help maintain reduced P_{O_2} in the water.

Physiological and behavioural measurements

Larvae placed within the flow-through chamber (refer to Table 1 for numbers) were visualised under high-power magnification (×40) using a binocular microscope (Leica MZ12, Leica Microsystems, Wetzlar, Hessen, Germany). Videos were made using a Lumenera Infinity 1 digital camera mounted onto the microscope and captured using Lumenera Infinity Capture Imaging Software (Lumenera Corporation, Ottawa, ON, Canada). Control larvae were kept in the flow-through chamber under normoxic conditions for 60 min and filmed for 30 s every 5 min (6.5 min in total). Larvae exposed to acutely declining P_{O_2} (~1.5% min^{-1}, total duration of exposure 1 h) were filmed for 30 s every 2 min. Heart rate of larval and true hearts was counted manually from videos. The two hearts were easily distinguishable from each other through their distinct position within the larva, with the larval heart lying on the floor of

Table 1. Number of egg masses (*N*) and larvae (replicates) used for each of the six developmental stages for control (white rows) and declining levels of dissolved oxygen (DO; grey rows)

	Larval heart		True heart		Spinning	
Day	*N*	Replicates	*N*	Replicates	*N*	Replicates
13	3	13			3	13
	3	15			3	15
15	3	10			3	10
	5	17			5	17
17	3	16	3	18	3	23
	3	15	3	15	3	15
19	3	18	3	19	3	19
	3	18	3	15	3	18
21	3	12	3	14	3	16
	3	19	3	19	3	19
22	3	19	3	17	3	19
	3	20	3	20	3	20

the mantle cavity (Fig. 1, Fig. 2A,B) and the true heart located beneath the right posterior dorsal surface of the shell (Fig. 1, Fig. 3C,D). Rotational activity was expressed as the percentage of the 30 s interval spent rotating.

Morphological measurements

Velum area was measured by doubling the measure for one lobe made using ImageJ (Abramoff et al., 2004) from a single frame extracted from the movie; the image used was one in which the entire velum area was visible. In addition to measurements of the six developmental stages studied here, data for velum size ontogeny measured as part of a previous study (Ellis et al., 2009) were used.

Data analysis

Repeated measures ANOVA were used to test for differences in heart rate of larval and true hearts, and rotation rate between time intervals for both control and treatment larvae over the duration of the 1 h experiments.

RESULTS

Patterns of development

Fig. 4 shows micrographs of the different developmental stages investigated, together with data on changes in velum area, larval and true heart activity and rotational activity during larval development. Velar lobes were first observed in 4 day old larvae (Fig. 4A). At this time, the velum area was small (4440±305 μm^2, mean ± s.e.m.) but increased rapidly over the next 5 days, reaching a maximum in 10 day old larvae (34,015±2504 μm^2) (Fig. 4B). From day 10 onwards, velum area decreased steadily and the velum had disappeared by day 24 (Fig. 4B). The larval heart began to beat on day 13 (Fig. 2, Fig. 4A,C), at the time when the velum size began to decline. Larval heart beat rate then increased over the next 4 days, reaching a maximum in 17 day old larvae (66±1 beats min^{-1}), after which time its beat rate decreased rapidly (Fig. 4C). The larval heart disappeared 23–25 days post-laying, at roughly the same time as the velum (day 24) (Fig. 4C).

The true heart appeared 17 days into development (heart rate 55±1 beats min^{-1}) in most larvae (Fig. 3, Fig. 4A,C) and its rate of beating increased (to 82±1 beats min^{-1} on day 22) as that of the larval heart declined (Fig. 4C).

In all six developmental stages, larvae spent <10% of their time rotating and this was invariant with development (Fig. 4D).

Larval heart responses to acutely declining P_{O_2}

No change in heart rate was observed during the 1 h time period that individuals were maintained in control conditions in the chamber (Larval heart: day 13, $F_{12,125}$=0.60, P=0.841; day 15, $F_{12,37}$=0.10, P=1.000; day 17, $F_{12,149}$=0.30, P=0.989; day 19, $F_{12,146}$=0.29, P=0.990; day 21, $F_{12,60}$=0.67, P=0.771; day 22, $F_{12,39}$=0.42, P=0.946. True heart: day 17, $F_{12,176}$=0.18, P=0.999; day 19, $F_{12,136}$=0.38, P=0.970; day 21, $F_{12,102}$=0.40, P=0.960; day 22, $F_{12,77}$=0.56, P=0.868). Therefore, any changes in rate that we observed were attributed to the effect of declining P_{O_2}. Both larval and true hearts were broadly insensitive to acutely declining P_{O_2} down to a critical level, but there were subtle stage-related differences (Fig. 5A,B). Just after beating commenced (i.e. after 13 days), the activity of the larval heart was independent of external P_{O_2} (repeated measures ANOVA: $F_{34,240}$=0.60, P=0.963), with a mean rate of 56±2 beats min^{-1}. Two days later, however, heart

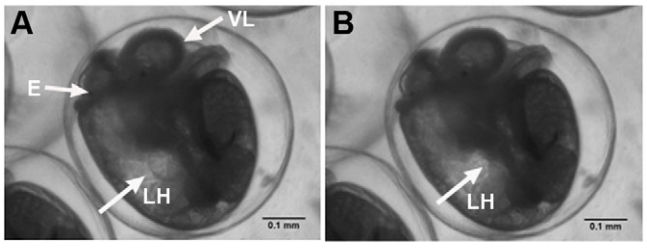

Fig. 2. The location and appearance of the larval heart in *L. obtusata* larvae 13 days after laying and before the appearance of the true heart. Larval heart (LH) is (A) 'inflated' and (B) 'deflated'. E, eye; VL, velar lobe.

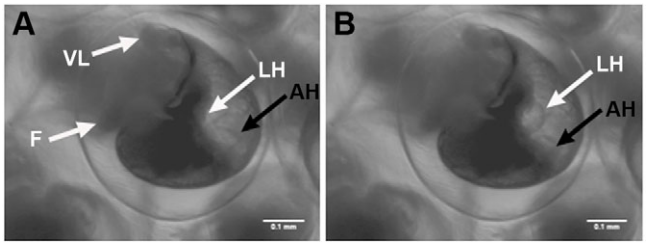

Fig. 3. The location and appearance of the larval heart in *L. obtusata* larvae 18 days after laying. Larval and true (adult) hearts are labelled LH (white arrow) and AH (black arrow). (A) 'Deflated' larval heart and 'inflated' true heart. (B) 'Inflated' larval heart and mostly 'deflated' true heart. F, foot; VL, velar lobe.

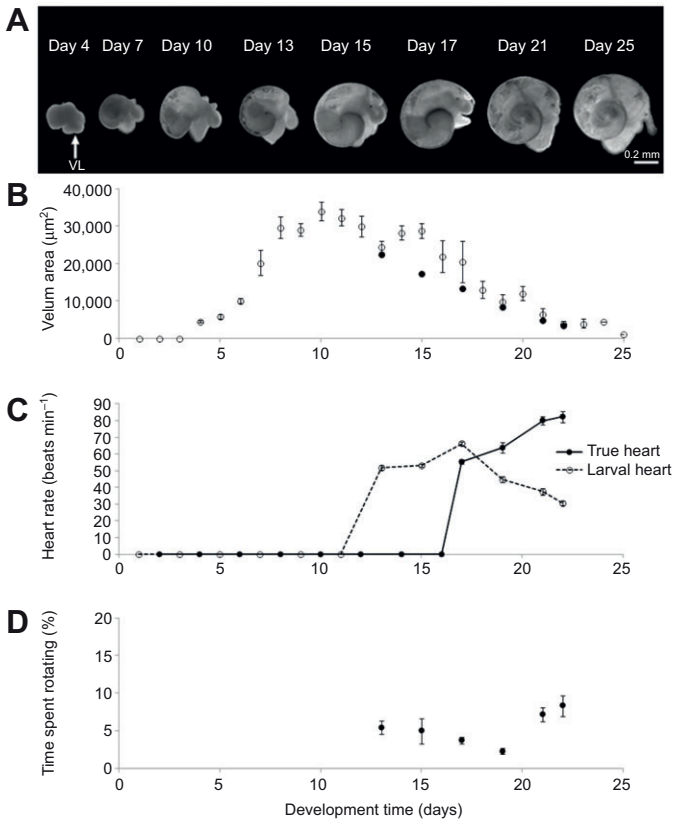

Fig. 4. Morphological and physiological development of *L. obtusata* between laying and day 25 (temperature 15°C, salinity S=34, P_{O_2} ~20 kPa). (A) Micrographs of different developmental stages. VL, velar lobes. (B) Velum area. (C) Larval and true heart activity. (D) Rotational activity. All values are means ± 1 s.e.m.

rate increased significantly during exposure to acutely declining P_{O_2} (from 57±3 beats min^{-1} at 100% saturation to 63±2 beats min^{-1} at 60% saturation; $F_{1,26}$=4.63, P=0.041), but decreased markedly below 40% saturation. At day 17, there was also a significant increase in heart rate ($F_{1,26}$=4.22, P=0.038), which was sustained until a P_{O_2} of around 20% saturation, i.e. between days 15 and 17, the P_{O_2} at which regulation breaks down and heart rate starts to decrease with decreasing P_{O_2} (the critical O_2 tension or P_c point). For days 19, 21 and 22, the heart rate of the larval heart was largely independent of external P_{O_2}, and it seemed that on day 19 and 21 the lower overall rate of beating was accompanied by a much lower P_c (<20% saturation).

True heart responses to acutely declining P_{O_2}

At days 17 and 19, true heart activity was independent of external P_{O_2} over the range investigated (day 17, $F_{33,277}$=0.68, P=0.906; day 19, $F_{31,217}$=0.69, P=0.890). However, in 21 day old larvae, heart rate increased significantly with acutely declining P_{O_2}, e.g. from 73±2 beats min^{-1} at 100% saturation to 85±3 beats min^{-1} at 60% saturation ($F_{1,19}$=10.55, P=0.004). The P_c was around 40% saturation. In 22 day old larvae, a similar overall pattern as for the 21 day heart was present, but the P_{O_2}-related increase in cardiac activity was of a greater magnitude and the P_c was lower, at around 60% saturation.

Rotational responses to declining P_{O_2}

There was no significant difference through time in the rate of rotation of control individuals (repeated measures ANOVA day 13, $F_{12,156}$=0.99, P=0.0.461; day 15, $F_{12,91}$=1.71, P=0.077; day 17, $F_{12,285}$=0.67, P=0.779; day 19, $F_{12,234}$=1.06, P=0.398; day 21, $F_{12,191}$=1.27, P=0.237; day 22, $F_{12.147}$=1.80, P=0.052). However, larvae of all six developmental stages increased the time rotating as P_{O_2} declined (Fig. 5C and Table 2). Thirteen day old larvae increased the time spent rotating the most, i.e. rotating for 91±3% of the time at 20% O_2 saturation. With further development, the increase in time spent rotating was reduced (i.e. time spent rotating at 20% saturation on day 15, 52±16%; day 17, 46±7%; day 19, 21±7%; day 21, 12±5%; day 22, 21±5%). Furthermore, the P_{O_2} at which the increase in rotation time was first noted decreased as development progressed, i.e. an O_2-related increase in rotation was evident at around 80% saturation in day 13 larvae but declined to <20% saturation in day 21 larvae.

DISCUSSION

Velum development and larval heart function are not tightly coupled

The molluscan velum is used for locomotion and collecting food particles, and is also presumed to be the primary area for respiratory gas exchange (Werner, 1955; Fioroni, 1966). Thus, it is reasonable to suggest that a concurrent development of the velum and larval heart would provide a way for a developing mollusc to maximise the internal circulation of fluids to and from the velum. Yet, the velar lobes of *L. obtusata* began to appear 4 days into development and reached their maximum size at day 10, whereas the larval heart did not start to beat until day 13 and its maximum beat rate was on day 17. The late development of the larval heart in this species, appearing when the velum area is already declining, does not seem to support earlier suggestions that the two structures are tightly linked functionally (Werner, 1955).

One reason for this apparent mismatch in the timing of velum and larval heart development in *L. obtusata* could be its encapsulated developmental mode. Indeed, the larval heart is known to develop long before the velum reaches its maximum size in caenogastropods with a planktonic veliger phase (Kohn, 1961; D'Asaro, 1965; Page, 1994; Dickinson and Croll, 2003; Gittenberger, 2003; Harding, 2006) and in planktotrophic opisthobranch larvae (Switzer-Dunlap and Hadfield, 1977; Gibson, 2003). Hence, it may be that when the velum is required for locomotion in the water column there is a greater need for this structure to be linked to an efficient circulatory pump. Care must be taken, however, in making such assumptions as it could also be argued that movement within the egg capsule through the action of velar cilia is equally important for feeding (Moran, 1999) and, as such, is equally energy demanding. In fact, if the rotational activity associated with the velum is important for O_2 uptake then it might be argued that it is more important for encapsulated larvae. A more extensive assessment of the functional role of the velum and its link with the circulatory system in developing molluscs, particularly those with planktonic larval stages, is required.

Appearance of a functional true heart coincides with peak larval heart activity

The true heart appeared 17 days into development in most larvae, coinciding with the time of greatest larval heart activity. The heart rate of the true heart increased as that of the larval heart decreased. This pattern is consistent with a shift from the larval heart driving general circulation to that role being taken over by the true heart. From day 17 on, normoxic true heart activity increased whereas that of the normoxic larval heart decreased with further development. While the idea of an extracardiac structure ceasing to function just

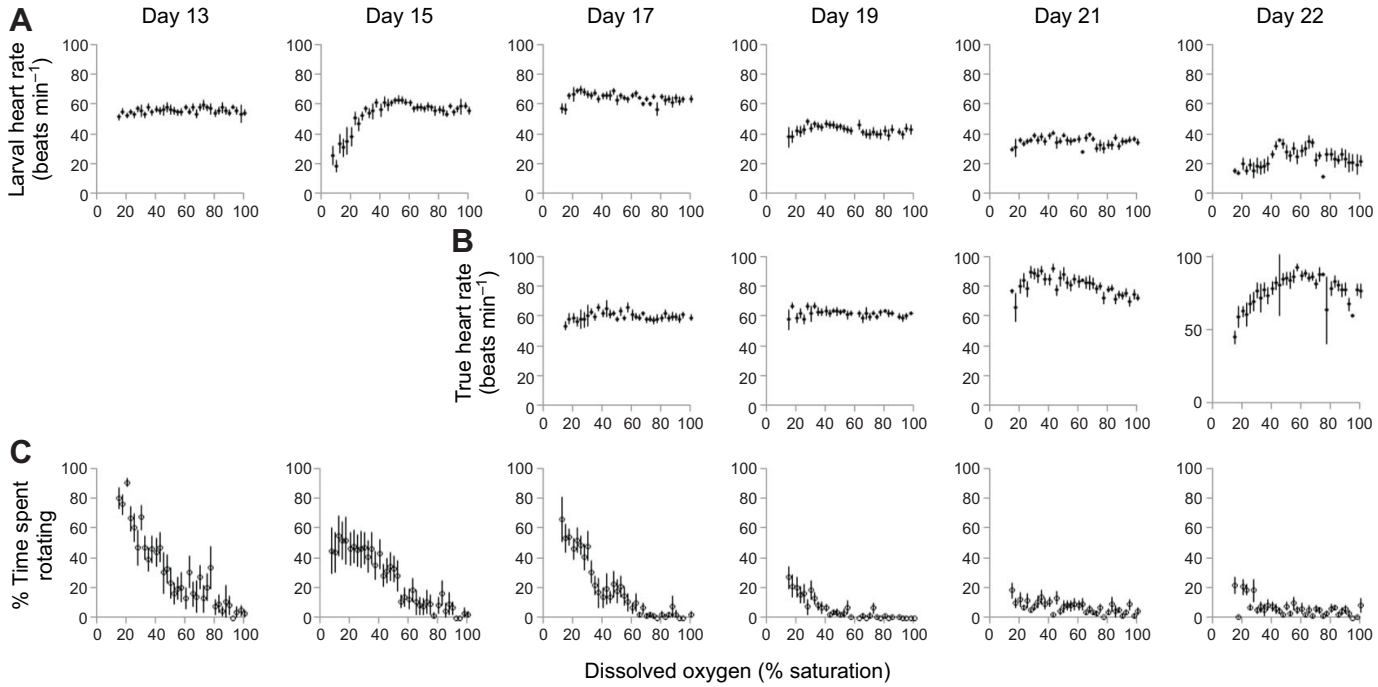

Fig. 5. The effect of acutely declining P_{O_2} on the activity of the larval heart (A) and true heart (B), and on rotational behaviour (% time spent rotating; C) in six different developmental stages of *L. obtusata*. For the number of individuals in each experiment refer to Table 1. A dissolved oxygen saturation of 100% is equivalent to 8.565 mg l^{-1} (Weiss, 1970).

as the true heart comes on line is not novel (Spicer, 2006), this is the first time it has been demonstrated for molluscs.

Regulation of the true heart during exposure to declining P_{O_2}

Shortly after the onset of beating (day 17) the true heart was insensitive to acutely declining P_{O_2}. This cardiac insensitivity to hypoxia in early larval stages has also been observed in a number of invertebrate (Reiber et al., 2000; Harper and Reiber, 2006) and vertebrate (Fritsche and Burggren, 1996; Jacob et al., 2002) species and has been interpreted as the absence of cardiovascular regulation. However, by day 21 we see the emergence of a heart that can increase its rate of beating in response to acutely declining P_{O_2}. This could be interpreted as the appearance of cardiac regulation. Interestingly, while the responsiveness to acutely declining P_{O_2} was greater in the oldest developmental stage investigated (day 22), the P_c was higher, meaning that at the earliest stage at which regulation was observed, the response occurred at a much lower P_{O_2} than in the slightly older individuals. Studies on the respiratory development of brine shrimp *Artemia franciscana* (Spicer, 1995a; Spicer and El-Gamal, 1999) and Norway lobster, *Nephrops norvegicus* (Spicer 1995b; Spicer and Eriksson, 2003), show a decrease in P_c with development (O_2 uptake) whereas the only study of respiratory development in a cephalopod mollusc species showed the opposite (Wolf et al., 1985). It has been suggested that the P_c is determined primarily by the amount of O_2 available to an animal in its environment (Spicer and Rundle, 2007). In the case of the lobster, the decrease in P_c during development was linked to the ecological transition from an O_2-rich planktonic habitat to a potentially O_2-limited fossorial, benthic habitat. In the case of the cephalopod, the ecological shift was the other way round, i.e. from a benthic to a planktonic habitat. Nevertheless, once the ability to regulate cardiac activity in the face of declining P_{O_2} is established, it is broadly similar to the patterns observed for adult molluscs (Bayne, 1971; Brand and Roberts, 1973; Taylor, 1976; Booth and Mangum, 1978; Dieringer et al., 1978; Marshall and McQuaid, 1993; Polhill et al., 1996).

Table 2. Results of repeated measures ANOVA testing for the effect of declining P_{O_2} on time spent rotating for each day of development

Day	d.f.	*F*	*P*	R^2 (%)
13	30	9.76	<0.001	40.91
15	29	5.65	<0.001	29.29
17	29	10.94	<0.001	42.97
19	23	4.16	<0.001	18.37
21	21	2.06	<0.01	7.2
22	17	2.84	<0.001	8.3

Regulation of the larval heart during exposure to declining P_{O_2}

Just because the activity of the true heart of *L. obtusata* is able to increase in response to declining P_{O_2} does not necessarily mean that the larval heart can do the same. That said, what is remarkable is that, broadly speaking, it does do the same. Having been insensitive to P_{O_2} on day 13, by days 15 and 17 larval heart activity is able to increase in response to declining P_{O_2}. As was the case with the true heart (above), this could be interpreted as evidence of regulation. If so, this is the first evidence for the larval heart being subject to regulation like the true heart. However, after day 17 when the larval heart showed its greatest activity (in terms of beating), the responsiveness of the larval heart to P_{O_2} seems to diminish, which could be interpreted as a loss or breakdown of regulatory ability. Consequently, we suggest that larval heart activity, in common with the true heart, can be actively regulated to some extent by the snail (at least in response to P_{O_2}). In both cases, this regulation develops a few days after the hearts begin to beat. In the case of the larval

heart the cessation of regulation coincides with the appearance of regulation by the true heart. So, we go further and posit the hypothesis that control of cardiovascular activity in larval *L. obtusata* passes from one distinct structure to another, from larval to true heart, and that the time of this transition is not coincidental.

Rotational activity during exposure to declining P_{O_2}

In addition to changes in heart activity, larvae were able to respond to declining P_{O_2} with a dose-dependent increase in time spent rotating (Fig. 5C). There were subtle differences in this response between developmental stages. Thirteen day old larvae increased the time spent rotating the most (i.e. rotating for 91±3% of the time at 20% saturation). With further development, both the maximum amount of time spent rotating and the P_{O_2} at which the increase in rotation time was first observed decreased (Fig. 5C). This decline in activity at low P_{O_2} between days 13 and 22 coincided with the gradual re-absorption of the velum and its consequent decrease in size. At present, it is unclear whether the decrease in rotational activity with further development is mainly a result of the decrease in size of the velum or the gradual loss or breakdown of its regulatory ability, although it is likely that both of these factors play a role.

Rotational activity, which is driven through cilia located on the velum, has been suggested to mix intracapsular fluids and thus facilitate diffusion of O_2 into the egg capsule (Kuang et al., 2002; Goldberg et al., 2008; Byrne et al., 2009). The rotational activity of *L. obtusata* larvae therefore potentially played an important role in maintaining O_2 uptake as P_{O_2} declined, with the velum contributing to extracardiac cardiovascular function. We suggest that rotational activity at different developmental stages is related to the ontogeny of the larval heart. At 13 days, larvae possessed a larval heart that was not able to actively respond to declining P_{O_2}; however, they were able to markedly increase the time spent rotating at this stage of development. Thus, O_2 provision may have mainly been adjusted through the function of the velum, compensating for the lack of regulation of the larval heart. At days 15 and 17, larvae spent little time rotating before the bradycardia of the larval heart was induced, but markedly increased the time spent rotating once the activity of the larval heart began to drop (Fig. 5A,C), suggesting that the function of maintaining O_2 uptake with decreasing P_{O_2} was met by the activity of both the velum and the larval heart, with rotational activity becoming more important at low P_{O_2}. Consequently, our data support the view that in early larval stages, the velum-driven, rotational activity fulfilled the function of maintaining O_2 uptake as P_{O_2} decreased and, with further development, there was a gradual transition where this function was passed from one transitory structure to another – from the velum to the larval heart. As we hypothesised for the transition of cardiovascular function from larval heart to true heart, the timing of this transition is not coincidental.

Concluding remarks

Most of our knowledge of invertebrate cardiovascular physiology derives from adult organisms. The way that cardiovascular function develops and changes during development is poorly understood in most groups (McMahon et al., 1997a; McMahon et al., 1997b). With the exception of two very old studies that investigated the effect of temperature on the heart rate of freshwater pulmonates (Bachrach and Cardot, 1923; Cardot, 1924), there is almost no literature on developing molluscs (McMahon et al., 1997a; McMahon et al., 1997b). Yet, we know from other groups where the development of the invertebrate cardiovascular system has been investigated that it often matches, or even exceeds, the complexity of the adult system (McMahon et al., 1997a). This is perhaps not surprising when considering the marked morphological and physiological changes that the larvae often go through during development. Additionally, invertebrate larvae are frequently exposed to changes in biotic and abiotic environments. The cardiovascular system must fulfil and maintain its important function in the face of such environmental challenges and whilst many structures and regulatory systems are still developing.

One of the main challenges for encapsulated embryos, such as *L. obtusata*, is probably O_2 supply, as embryos are often crowded within egg masses and the jelly matrix around the eggs limits O_2 diffusion (Brante et al., 2009). Furthermore, intertidal habitats are subject to large P_{O_2} fluctuations (Truchot and Duhamel-Jouve, 1980; Agnew and Taylor, 1986) and embryos in egg masses have no possibility of avoiding hypoxic areas once the egg mass has been deposited in one location and can therefore be exposed to hypoxic conditions for longer periods. The three structures studied here, the velum and the larval and true hearts, all showed responses to hypoxia and potentially play a role in the O_2 provision for larvae. However, they do so in different ways. The velum constitutes a large surface area for gas exchange (D'Asaro, 1965) and, additionally, drives a rotational response under low O_2, thus enhancing O_2 diffusion into the egg capsule (Goldberg et al., 2008). The larval heart is anatomically connected to the velum and therefore distributes O_2 within the larva, assisting the true heart with internal circulation. The cardiovascular function in developing *L. obtusata* is therefore achieved through different means and, based on our results, we argue that there is a change of strategy and the structures involved throughout development. We hypothesise that O_2 delivery through the velum might suffice in early developmental stages, when the larvae are still small (and therefore the diffusion distance is shorter than later on in development). With further development, consequent increases in size and metabolic demands of the larvae as well as progressing shell calcification, internal circulation through the larval heart becomes more important.

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