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Journal of Experimental Marine Biology and Ecology 329 (2006) 144–152

Journal of EXPERIMENTAL MARINE BIOLOGY AND ECOLOGY

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Position of egg within a clutch is linked to size at hatching in a demersal tropical fish

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Received 9 May 2005; received in revised form 25 August 2005; accepted 26 August 2005

Abstract

Size variation among propagules is ubiquitous and small initial differences in size can be critical to survival, particularly in taxa where initial survival is variable and strongly size-dependent. Despite this, the sources of size variation among fish at hatching are rarely investigated. This study examined spatial position within egg clutches as a source of size variation at hatching of the benthic spawning fish *Amphiprion melanopus*. We quantified within-clutch size variation at hatching and found that newly hatched larvae from the periphery (5 mm from edge) of 2-dimensional clutches were smaller in standard length, cranial depth, eye diameter and body area (7%, 8%, 4% and 11%, respectively) than larvae from the interior positions within clutches. To investigate the source of this variation, sizes of embryos at different locations with clutches were measured within 2 h of fertilisation (8 d before hatching). Newly laid embryos from the clutch periphery were smaller in length and volume than embryos from the clutch interior (>2% and 4–6%, respectively). These eggs from the periphery also had a 33% lower rate of oxygen consumption than did embryos from the clutch interior, throughout development. The relationships between position within a clutch and egg size, oxygen consumption and larval size imply that size variation in larval fish at hatching is partly generated during early embryogenesis, either from maternal endowment or maternal nest design, and was amplified throughout development.

Keywords: Coral-reef fish; Development; Maternal effect; Micro-optode; Respiration

1. Introduction

Initial propagule size is a critical determinant of survival in many organisms (Forester, 1979; Beacham and Murray, 1985; Kaplan and King, 1997). Within a variety of oviparous vertebrates, including frogs (Kaplan and King, 1997), fish (Beacham and Murray, 1985), salamanders (Forester, 1979), lizards (Sinervo, 1990), and birds (Price, 1998) differential maternal investment in egg size within a clutch results in variation in hatchling size. In marine fishes, size variation exists at hatching (Kerrigan, 1997) and large size often confers an advantage in the post-embryonic stages (Miller et al., 1988; Chambers and Leggett, 1996; Vigliola and Meekan, 2002; McCormick and Hoey, 2004). Early size advantages in marine fish can be cumulative and may be amplified throughout development (Vigliola and Meekan, 2002). Despite this, the environmental conditions and physiological processes during embryogenesis (i.e. life stages prior to hatching) are seldom considered as a source of maternally derived size variation critical for post-embryonic growth and survival (Bernardo, 1996a).

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doi:10.1016/j.jembe.2005.08.012

In fish, demersal eggs do not typically disperse. Consequently, embryos develop in or near the parentally chosen environment for the duration of the embryonic period. The embryos are susceptible to a range of conditions in their surrounding environment, yet have little ability to regulate their environment (Blaxter, 1969). For example, rates and synchrony of embryo development may be affected by oxygen concentration which in turn may vary with maternally derived factors such as nest site and clutch configuration, including size, density and shape, in fish (Jones and Reynolds, 1999) and other taxa (e.g. gastropods, Lardies and Fernández, 2002; Cancino et al., 2003; cephalopods, Cronin and Seymour, 2000; Steer et al., 2003; fishes, and amphibians, Pinder and Friet, 1994). Thus, limited oxygen availability due to nest design can restrict the rate of development of individuals near the centre of an egg clutch and lead to asynchronous development of embryos.

There are three likely sources of variation in the development of demersal embryos: maternal allocation (e.g., genetic differences, egg quality and clutch configuration); local environment (e.g., heat and gas exchange and clutch configuration); and in some taxa, parental maintenance of the embryos, which can modify the local environment by parental fanning of the clutch (Green and McCormick, 2005). Thus, the size of larvae hatching from benthic eggs is likely to be the result of the maternal allocation, subsequently modified by the propagule's interactions with its environment (Bernardo, 1996b). However, the relative roles of maternal influences and position within the clutch, on embryonic development have not been examined in detail in marine fishes.

Here, we investigate the variability in maternal allocation to eggs within clutches of the tropical anemonefish *Amphiprion melanopus*. We then examine the effect of an egg's position within clutches on rates of oxygen consumption of embryos (a proxy for metabolism) and the persistence of initial size differences among embryos throughout development. This study provides the first detailed examination of the relative importance of within-clutch position to the growth of embryos in a demersal fish.

2. Materials and methods

2.1. Study species and preparation of egg clutches

In nature, pairs of *A. melanopus* attach 300 to 700 capsule-shaped eggs in single-layer circular clutches (40–100 mm diameter) to the benthos in semi-cryptic habitats within the periphery of their host anemone. The parents tend these clutches of eggs until the embryos hatch 7.5 d after fertilisation, with well-developed skeletal, organ and sensory systems (see Green and McCormick, 2001 for comparisons to other fish species). Embryogenesis occurs at the parentally chosen nest site and energy for embryogenesis comes entirely from the maternal allocation to her ova. These features of maternal allocation, nest site selection and clutch attendance by parents make *A. melanopus* an ideal model for investigating the roles of maternal contribution and clutch position in size variation of larvae at hatching.

Pairs of adult *A. melanopus* were captured from the northern section of the Great Barrier Reef, Australia, (16°8′ S, 145°7′ E), and maintained in 70-l flow-through tanks at the James Cook University Research Aquarium. Broodstock pairs were provided hollow cement blocks (internal dimensions $16 \times 16 \times 19$ cm) for nesting, and were conditioned to lay their clutches (similar size and dimensions to wild clutches) onto roughened acetate sheets attached to the inside of the nests.

2.2. Oxygen consumption measurements

Seven clutches of eggs from different broodstock pairs were removed from parental care in order to measure oxygen consumption throughout development. Each egg clutch was photographed against a scale bar to determine clutch size and embryo densities using image analysis (Image Tool, UTHSCSA, San Antonio, USA). Once a clutch was removed from the parents, it remained in the experimental chamber as parent fish often eat their eggs if they are re-introduced (B. Green, pers. obs.).

The effects of within-clutch position of embryos on their rates of oxygen consumption were assessed using a boundary-layer profiling technique (e.g. Kuhl et al., 1995). This method quantifies the flux of oxygen through the semi-stagnant boundary layer above a respiring surface by taking vertical profiles of oxygen and estimating the change in oxygen concentration using Fick's first law of diffusion (Eq. (1)). Using this equation, the flux (J, μmol cm^{-2} s^{-1}) of oxygen through the boundary layer, and thus the rate of consumption of oxygen by embryos within the clutch, was estimated as

$$J = -D\frac{\partial[O_2]}{\partial_x}, \quad (1)$$

where D is the diffusion coefficient (cm^2 s^{-1}), $[O_2]$ is oxygen concentration (μmol L^{-1}) and x is the vertical position within the boundary layer (mm, measured from

the tip of the eggs). The upper limit of the boundary layer was estimated as the point at which oxygen concentrations approximate mainstream values (Patterson, 1992). The oxygen diffusion coefficient (D) was determined by the slope of the O_2 concentration versus x profile near the egg surface (e.g. see Epping et al., 1999). Oxygen consumption rates for each clutch were standardised for embryo density in order to compensate for differences in clutch size and density.

Oxygen consumption measurements were taken within an open flume channel (Fig 1., L 370 mm, W 151 mm, H 68 mm) similar to that described by Patterson et al. (1991). Water flow of 3.5 cm s^{-1} was generated by a submerged bilge pump (Johnson, 1000 W) attached to a variable, regulated power supply. Flow straighteners at either end of the channel minimised turbulence. Individual acetate sheets with an egg clutch attached were clamped onto the channel floor approximately 150 mm downstream of the flow straighteners. Flow velocity was measured using visual tracking of neutrally buoyant particles over a known distance, and laminar flow was visually checked by injecting milk into the water current at the upstream end of the chamber. Once the boundary layer had formed over an egg clutch (approximately 30 min), profiles of oxygen concentrations were determined at seven positions across the maximum diameter of the clutch perpendicular to the flow direction (Fig. 1) using a micro-optode (140 μm tip, Presens, Germany, see Klimant et al., 1995) supported by a micromanipulator (MM-33, Sutter) and positioned with an accuracy of 5 μm. Vertical profiles of oxygen concentrations were measured above the eggs at 0.2 mm increments (Klimant et al., 1995), within a minimum of 50 readings of O_2 concentration taken per vertical profile. The micro-optode was calibrated daily at 100% oxygen saturation and 0% oxygen saturation (water supersaturated with sodium sulphite zero DO_2 solution). Calibration was periodically checked throughout the measurement series. All oxygen measurements were carried out with automatic temperature compensation (ambient water temperature was stable at 28 ± 0.5 °C). Not all clutches were measured on every day as the flow chamber restricted simultaneous assays of clutches, however 3 to 4 clutches were assayed for each day of development (days 1 to 7, see Green, 2004 for details). Oxygen measurements were taken at the same time every day, commencing at 0900 h.

2.3. Propagule size within a clutch

In order to examine size variation of embryos and larvae within a clutch, embryos were sampled at two developmental stages from 8 egg clutches in addition to the 7 clutches assayed for O_2 consumption described above. Four clutches were sampled within 2 h of being laid in the nests (day 1) and the remaining four clutches were maintained by parents until hatching and then measured. Each of the 4 clutches of embryos sampled at day 1 was divided into two positions: periphery (5 mm from the edge) and interior (the remaining embryos), (Fig. 1). Day 1 samples were immediately preserved in Marine Bouins fixative for later measurement. Size of newly hatched larvae was

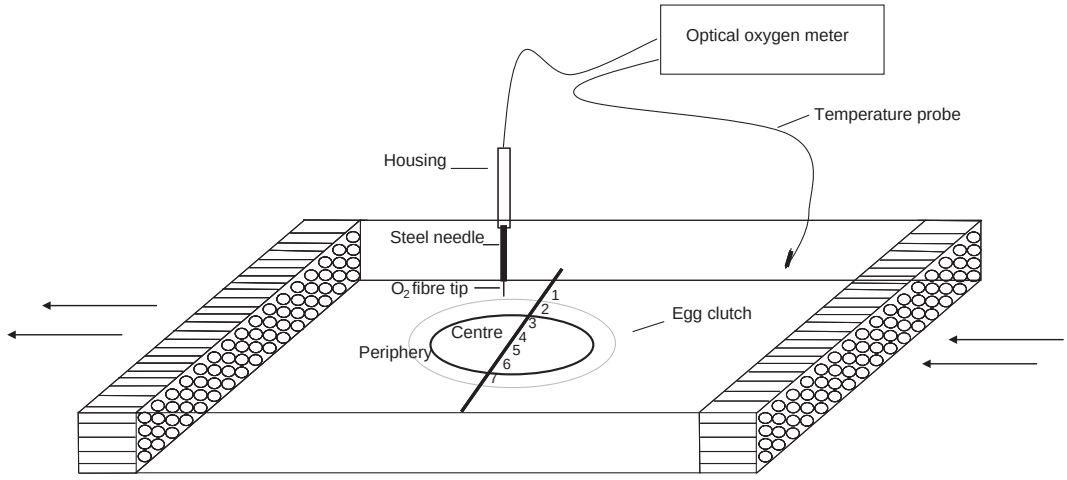

Fig. 1. Schematic representation of a benthic egg clutch attached to the floor of the laminar flow chamber. The micro-optode used to sample vertical oxygen profiles from the boundary layer above developing fish embryos is represented. Arrows indicate the direction of the water flow within the chamber. Positions of oxygen profile are numbered; 1 and 7 represent measurements at the clutch periphery, all other numbers represent the clutch interior.

also determined for embryos drawn from the clutch periphery and interior by removing late stage embryos with a scalpel 30 min prior to their expected time of hatching. The embryos from each location were then placed in a 500 ml beaker with sea-water and aerated in darkness for 1 h, simulating dusk (their natural hatching time). Once hatched, all larvae were then preserved in Marine Bouins fixative for later measurement. Each clutch was sub-sampled and 20 embryos or larvae from each position (periphery and interior) were photographed on a dissecting microscope with a camera attachment. Images were captured and measured using Image Tool. No correction was made for shrinkage due to preservation. For day 1 samples, egg length, maximum egg width and yolk area were measured on all captured images. Egg volume was approximated using the formula for a cylinder: volume $=\pi\ r^2\ L$, where r is egg radius and L is egg length. A sub-sample of eggs from the periphery ($n=10$) and interior ($n=10$) was blotted dry and weighed on a Meitler balance (model AE 240) to 0.01 mg accuracy, to provide standardisation of respiration per unit mass. Standard length, eye diameter, cranial depth and body area (excluding fins) were measured on newly hatched larvae. Although eye size is not expected to change in response to environmental conditions (Fuiman et al., 1998), it provides a standard for comparison with changes in the other variables.

Lastly, we compared rate of oxygen consumption standardised to average size of newly laid embryos (day 1) to determine if the difference in measured rates of respiration was a function of egg size alone. Average respiration rates for the peripheral embryos (position 1 and 7, Fig. 1) and interior embryos (all other positions where respiration was measured) were grouped from all egg clutches and standardised for the average size of embryos from the periphery and the interior of the clutches for day 1.

2.4. Data analysis

The diffusion coefficient (D, Eq. (1)) and associated standard errors for each trial were estimated using linear, least-squares regression within the initial and approximately linear portion of the profile. The r^2 value for each D estimation was ≥ 0.85. Two-way ANOVA was used to test for the effect of egg age and position within a clutch on the rate of oxygen consumption. Data were transformed (square root) to achieve variance homogeneity and normality. MANOVA was used to test for the effect of individual clutch and position within a clutch on variables at hatching. Post hoc test were performed by Tukey's HSD multiple comparisons.

3. Results

3.1. Initial embryo size

On the day eggs were laid and fertilised (day 1), embryos from the clutch periphery were significantly smaller ($\approx 2\%$ in length, 3% volume) than embryos in the clutch interior (Table 1). This difference in egg volume was small but consistent between 3 of the 4 clutches measured (Fig. 2a). Yolk area, a measure of yolk quantity, which is the source of nutrition for the embryos for the following eight days of development, did not vary significantly between the periphery and interior of the egg clutches (Fig. 2b, Table 1). Measures of yolk and egg size varied significantly among clutches indicating inter-female differences (Table 1).

Table 1
Effects of clutch identity and position within a clutch on size attributes of day 1 embryos (two-way ANOVA) and larvae at hatching (MANOVA)

Factor: day 1	Source of variation	*df*	MS	*F*	*p*
Egg volume	Position	1	0.451	10.7	0.001
	Clutch	3	0.897	21.2	<0.001
	Position × clutch	3	0.096	2.3	0.080
	Error	232	0.042		
Yolk area	Position	1	0.029	1.6	0.208
	Clutch	2	0.269	14.7	<0.001
	Position × clutch	2	0.004	0.23	0.798
	Error	153	0.018		
MANOVA: hatching	Source of variation	*df*	Pillai's trace	*F*	p
	Position	4, 149	0.262	13.25	<0.001
	Clutch	12, 453	0.317	4.48	<0.001
	Position × clutch	12, 453	0.155	2.06	0.018

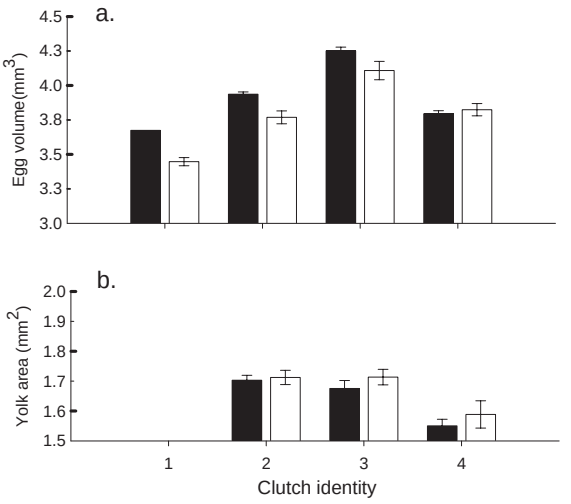

Fig. 2. Comparison of mean egg attributes (±se, $n=160$ eggs) between the interior (black bars) and periphery (white bars) of four clutches of *A. melanopus* sampled on the day that eggs were laid. (a) Egg volume; (b) mean yolk area (three clutches sampled only).

3.2. Size at hatching

Newly hatched fish sampled from the periphery of the clutch were generally smaller than fishes sampled from the clutch interior. Specifically, body length, body area (excluding fins), eye diameter and cranial depth were 7%, 11%, 4%, and 8% smaller on larvae from the periphery of three of the four clutches sampled (Fig. 3a, b, c, Table 1). There was no size difference between these positions in larvae from 1 of the four clutches measured.

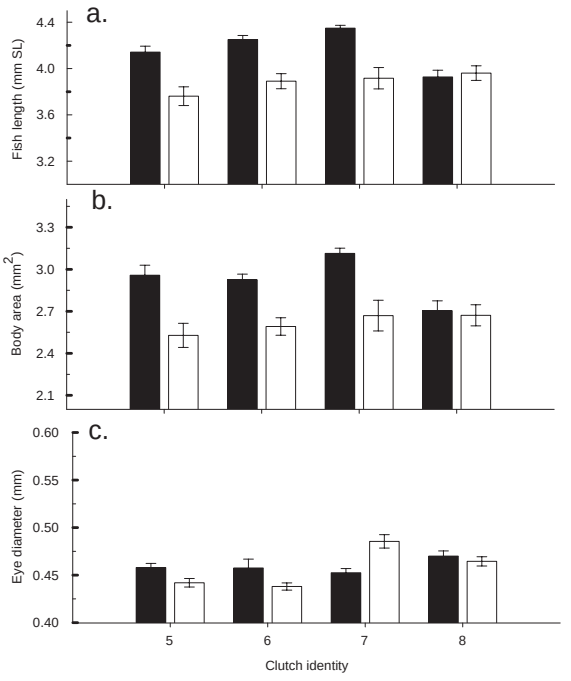

Fig. 3. Comparison of mean larval attributes (±se, $n=160$ larvae) of *A. melanopus* sampled immediately after hatching between fish originating from the interior (black bars) and periphery (open bars) of the clutch, for 4 separate clutches. (a) Standard length (SL); (b) body area; (c) eye diameter.

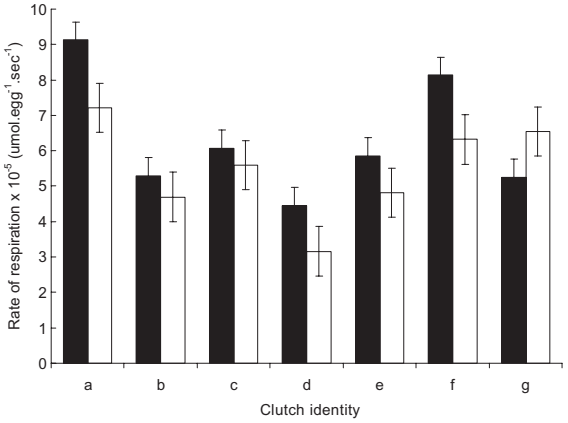

Fig. 4. Comparison of mean rate of oxygen consumption (±se) of *A. melanopus* embryos measured at the interior (black bars) and periphery (open bars) of the clutch averaged throughout development for 7 egg clutches. Values are standardised for embryo density.

3.3. Oxygen consumption

At distances >5 mm from the periphery of the clutch, the rate of respiration was not different from the respiration rates for all 5 positions measured from the clutch interior (Tukey's HSD, $p>0.05$), consequently all positions >5 mm from the edge will be referred to as interior throughout this manuscript. The density of egg clutches ranged from 1.9 to 6.0 embryos mm^{-2} and the average density was 3.6 embryos mm^{-2}. When respiration rate throughout development was standardised to embryo density (and initial average embryo size), peripheral embryos (position 1 and 7, Fig. 1) had a 33% lower respiration rate than interior embryos,

Table 2
The effect of egg age and position within a clutch on the rate of oxygen consumption standardised for embryo density within a clutch and egg size on day 1

Source of variation	*df*	MS ($\times 10^{-6}$)	F	*p*
Egg age	6	43	7.70	<0.001
Position within a clutch	1	30	5.45	0.020
Egg age × position	6	5	0.95	0.460
Error	267	6		

Data were transformed (square root) to achieve homogenous variances.

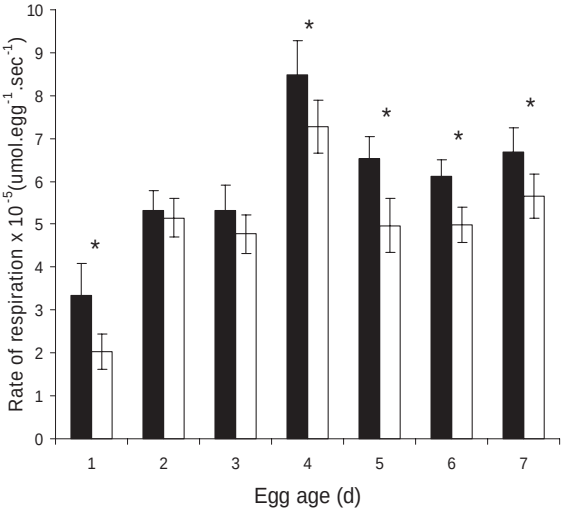

Fig. 5. Comparison of mean rate of oxygen consumption (± se) within 6 *A. melanopus* embryo clutches throughout development, measured at the interior (black bars) and periphery (open bars). Clutch g. (Fig. 4), where differences between periphery and interior were reversed is not included. Values are standardised for embryo density. *Denote significant differences Tukey's, $p<0.05$.

2.89×10^{-5} compared to 4.31×10^{-5} μmol O_2.egg^{-1} s^{-1}, although this pattern did not hold for one out of seven clutches (Fig. 4, Table 2). Differences in respiration rate between the interior and peripheral embryos occurred throughout development (Fig. 5).

When respiration rate was standardised to mean egg mass at the clutch periphery and interior, respiration of peripheral embryos was 24% lower than interior embryos, 3.38×10^{-5} c.f. 4.42×10^{-5} μmol O_2 mg^{-1} s^{-1}. There was no effect of density on the rate of respiration.

Embryos from the clutch periphery and interior hatched at the same time, suggesting that neither initial state nor differential environmental conditions influenced developmental rate.

4. Discussion

4.1. Maternally derived propagule variation

Variation in the size of propagules within a clutch from the benthic spawning *A. melanopus* is initially maternally derived, either by resource allocation or nest configuration. The initial small differences in embryo size between the periphery and the rest of the clutch suggest that females may invest less into peripheral embryos or that nest configuration favours growth and metabolism (measured by O_2 consumption) in embryos away from the edge of the clutch. Regardless of which of these two (or other) mechanisms is operative, this advantage is apparent in embryo size within hours of being laid. While the initial size differences among embryos were small, they provided a growth advantage to non-edge embryos that increased throughout development and was apparent at hatching.

It is unclear whether initial embryo sizes are due to size differences at deposition or the differences occur within the first hours after being laid. Differences at deposition equate to differential maternal investment in egg size, and possible reasons for this include: 1) limited maternal resources; 2) adjustment to uneven distribution of predation risk for embryos within a clutch; and 3) reduction in competition among propagules through variation in propagule size (McGinley et al., 1987). The first mechanism assumes that propagule variation is undesirable while the final two suggest it is adaptive.

Numerous examples exist whereby limited female resources create size differences in embryos, however the pattern of variation is not predictable between species. For example, in newts, *Hynobious nigrescens* and *H. lichenatus* the female lays the largest eggs first and egg size is reduced as reproductive resources are depleted (Takahashi and Iwasawa, 1988). In the pied flycatcher, *Ficedula hypoleuca* (bird), females partition resources and lay the largest eggs last (Potti, 1993). In the present situation, female *A. melanopus* commenced laying their clutches at one end of the clutch and zigzag across the clutch, finishing at the opposite end (B. Green, pers. obs.), such that peripheral eggs were laid temporally interspersed with interior eggs. Consequently, the size of the eggs appears to be unrelated to the temporal pattern of laying. The distribution of smaller embryos around the periphery as reported here suggests that variation in embryo size is unlikely to be due to maternal resource limitation.

Risk of predation may be higher for peripheral eggs in a parentally protected egg clutch. Benthic spawning fishes spend much time and energy defending their nests against egg predators, such as wrasses and butterflyfishes (Tyler, 1995). In colonial nesting fishes, nests at the periphery of a colony face higher predation risk from egg predators than do central nests (Dominey, 1981; Foster, 1989). Similarly, in a near circular benthic clutch, nest-guarding parents have a higher likelihood of protecting central eggs from egg predators at the expense of peripheral eggs. Females could adapt to this differential risk by reducing energy investment to embryos at higher risk of predation; thereby producing smaller eggs on the periphery of an egg clutch. While eggs on the periphery of *A. melanopus* clutches were smaller compared to non-peripheral eggs, there was no

difference in the yolk quantity of embryos between locations. It appears that females are not producing wholly sacrificial embryos for the high risk periphery, therefore, the supposition that smaller eggs on the clutch periphery is an adaptation to reducing predation risk is not fully supported. Whichever mechanism is driving the differences in initial propagule size, the effects are increased throughout development and the resulting size differences at hatching may have ramifications for larval survival and recruitment success (Vigliola and Meekan, 2002).

4.2. Importance of within-clutch position

The position of an embryo within *A. melanopus* egg clutches was important to growth and oxygen consumption rate throughout development, regardless of clutch density. The mechanism driving this is unclear but it may be a response to gas exchange around the clutch, metabolites exchange between embryos, osmotic gradients, increased temperature due to a density effect at the clutch interior, or enhancement of initial size differences through size-related metabolism. It is clear however, that a size advantage is gained for embryos in the clutch interior. Diffusion of gases and the metabolic requirements of eggs within a clutch often constrain the size and shape of an egg clutch. For example, the structure and density of amphibian and marine invertebrate nests are critical to ensure that oxygen is available to the innermost eggs (Lucas and Crisp, 1987; Lee and Strathmann, 1998; Lardies and Fernández, 2002). Growth and development within an egg clutch vary in accordance with oxygen supply to eggs in a range of organisms. Peripheral individuals develop faster than more central embryos in three-dimensional gelatinous egg clutch of gastropods (Chaffee and Strathmann, 1984), some marine fishes (Giorgi and Congleton, 1984) and frogs (Salthe and Mecham, 1974) as oxygen diffusion is restricted to the central areas of these egg clutches. Egg position is important to normal development and survival of squid embryos, which are laid in strands and develop with no parental care. Proximal squid embryos develop slower and suffer higher mortality than their distal siblings at the outer perimeter of the egg mass (Steer et al., 2003). We had expected *A. melanopus* nests would reflect this general pattern of higher propagule growth and/or survival at the clutch periphery, however our results demonstrated the reverse pattern. This apparent pattern reversal may simply reflect the 2-dimensional structure of *A. melanopus* clutches which are more open to oxygen diffusion than 3-dimensional nests and receive enhanced oxygen replenishment through parental fanning (Green and McCormick, 2005).

4.3. Variation in size at hatching

Embryos of *A. melanopus* from the interior of egg clutches hatched out at larger sizes than individuals from the periphery. Size differences from the day of embryo deposition were enhanced throughout development by the effects associated with their position within a clutch, resulting in larger larvae hatching from non-peripheral areas. As size-adjusted oxygen consumption was higher at the clutch interior compared to the periphery it is apparent that position per se had an influence on oxygen consumption and therefore metabolism, regardless of the size difference. Slower metabolism (indicated by lower oxygen consumption) of the embryos on the periphery would retard embryo growth emphasising initial size differences with development, since growth is constrained by metabolic rate. Therefore, clutch micro-environment appears to contribute, in part, to increased metabolic rate, and is likely responsible for different growth rates throughout embryonic development within clutch position, resulting in size differences recorded at hatching.

All clutches did not show the same trends in offspring size and oxygen consumption, and the between-clutch variation in these variables was significant in this study. One clutch from each of day 1 and hatching showed little differences in size between the clutch periphery and interior, and one clutch from the oxygen assays did not have a gradient in metabolism. As each clutch came from a unique female these results suggest that there are two sources of variation in size of offspring: within-female differences as well as differences between-females. Size variation is largely of maternal origin, and differences between and within females are the key sources of such size variation (Chambers and Leggett, 1996). The differences in eye diameter between interior and periphery of the clutch were less consistent than the other morphometrics used. This is not unexpected, as the eye size generally does not respond to environmental variation (Fuiman et al., 1998). Embryos from the periphery and interior of the *A. melanopus* clutches hatched at the same time suggesting that neither initial size nor differential environmental conditions influenced developmental rate. The lack of a position affect on developmental rate is in contrast to observations on organisms in three-dimensional egg clutches where development is retarded in central embryos (e.g. gastropods Chaffee and Strathmann, 1984).

4.4. Relevance of laboratory measures to fields conditions

Anemonefishes show a high degree of parental care and nest-tending, oxygenating the eggs with fanning of the pectoral fins and adapting their fanning to the requirements of the embryos relative to ambient O_2 levels (Green and McCormick, 2005). Therefore, the laminar flow system employed for these measurements was quantitatively different to the conditions demersal eggs would experience in wild conditions, and more so in *A. melanopus* where the parental egg tending would create a turbulent flow regime. However the principle tested here was whether size variation is amplified during embryogenesis, and whether this was related to nest configuration and oxygen consumption. The artificial nests and flow regime allowed these factors to be quantified in a controlled environment. The experimental flow regime likely reflected flow rates in nature, as flow regimes are relatively stagnant in the semi-cryptic habitat that some cnidarians (which includes anemones) prefer (Patterson et al., 1991).

5. Conclusion

Our results suggest that position within the clutch is an important source of the high variation in larval size at hatching found within clutches of demersal eggs. Size differences at hatching were the product of the maternal allocation to the eggs, or maternally determined position within a nest and nest configuration, enhanced by influences from the clutch microenvironment. The size variation at hatching relative to position within the clutch in this study has implications for the survival of these larval reef fish. Evidence suggests that growing fast and being larger than average is an advantage that is increased throughout the larval period due to the correlation of size with a wide variety of performance attributes (Vigliola and Meekan, 2002; McCormick and Hoey, 2004). Large individuals from non-peripheral locations may have a greater probability of surviving due to their maternal endowment and embryonic environment and clutch position may be an important determinant of which individuals survive to later developmental stages.

Acknowledgments

We thank Peter Ridd for advice on hydrodynamics, and R.C. Chambers for constructive comments on the manuscript. This project was supported by an Australian Research Council Discovery grant to M.I.M. The work complies with the Australian Code of practice for the care and use of animals for scientific purposes and has approval AS 626. **[RH]**

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