Morphology, respiration and energetics of the eggs of the giant cuttlefish, *Sepia apama*
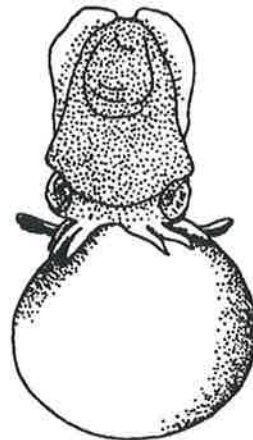

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Abstract

The giant cuttlefish (*Sepia apama*) of southern Australia lays among the largest molluscan eggs known. The eggs consist of a single embryo with attached yolk enclosed within a chorion membrane and wrapped in a series of jelly layers. Embryonic development requires 3-5 months to produce a miniature adult weighing 0.35 g, with an internal yolk constituting half of its dry mass. The growth pattern reflects the avian exponential growth model, however the growth rate is slower than in birds, indicating that metabolism is inherently low. Compounded with low incubation temperatures this prolongs the developmental period, which necessitates a greater degree of protection in the form of capsule material, which consists of 20% of the total egg energy content. Egg volume increases by 180% during development as a result of water accumulating in the perivitelline space, and this alters egg morphology and facilitates gas exchange. Oxygen diffusion across the capsule was modeled using the equation, $\dot{V}O_2 = GO_2 (PO_{2out} - PO_{2in})$ in which $\dot{V}O_2$ is the oxygen consumption rate, GO_2 is the capsule oxygen conductance, and the PO_2 values are the oxygen partial pressures across the capsule. During development $\dot{V}O_2$ rises exponentially as the embryo grows, reaching 5.5 $\mu l \ h^{-1}$ at hatching. Egg swelling causes GO_2 to increase, allowing maintenance of internal PO_2 high enough to allow unrestricted $\dot{V}O_2$ until shortly before hatching. Diffusion limitation of respiration in hatching-stage embryos is demonstrated by (1) increased embryonic $\dot{V}O_2$ when ambient PO_2 is experimentally raised, (2) greater $\dot{V}O_2$ of resting animals immediately after hatching, and (3) reduced $\dot{V}O_2$ of hatchlings at ambient PO_2 levels higher than internal PO_2 before hatching. Using direct calorimetry, the proportion of energy conserved in the hatchling was 61%. The amount of energy consumed during metabolism determined by direct calorimetry, was greater than that measured using respirometry. This suggests the embryo utilises anaerobic pathways for metabolism, which may be important when diffusion across the capsule limits the $\dot{V}O_2$. This occurs despite the

low temperature of incubation which extends the developmental duration, the inherently low metabolic rate and the high GO_2, indicating that *S. apama* eggs represent the upper boundary for molluscan egg size under natural environmental conditions.

Publications from this study

Cronin, E. R. and Seymour, R. S. (2000). Respiration of the eggs of the giant cuttlefish *Sepia apama*. Marine Biology (in press)

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Declaration

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

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Acknowledgments

Introduction

Sepia apama belongs within the class Cephalopoda, the most disparate class within the vast and diverse group comprising the second largest animal phylum, the Mollusca (Arnold 1971). The specialised cephalopod class is divided into three subclasses, the completely shelled Nautiloidea and Ammonoidea, and the Coleoidea in which the shell is internal and either reduced or absent (Fig. 1.1). All living cephalopods belong to this latter subclass, except for *Nautilus*, which is the sole extant shelled representative of the Nautiloidea. Within the Coleoidea the degree of modification of the shell has resulted in five extant orders. In the Sepioidea and Sepiolioidea (cuttlefish and sepiolids), the shell consists of a thickened plate with septa or is greatly reduced or absent, whereas the shell of Teuthoidea (squids) is reduced to a long flattened plate or 'pen', whilst in the Vampyromorpha and Octobrachia (octopus), the shell has completely disappeared.

Subclass	Superorder	Order	Suborder

Nautiloidea
(Family: Nautilidae)

Ammonoidea
(Extinct)

Coleoidea

Decapoda

Vampyropoda

Sepioidea

Sepiolioidea

Teuthoidea

Vampyromorpha

Octobrachia

Myopsida

Oegopsida

Cirroctopoda
(Cirrate)

Octopoda
(Incirrate)
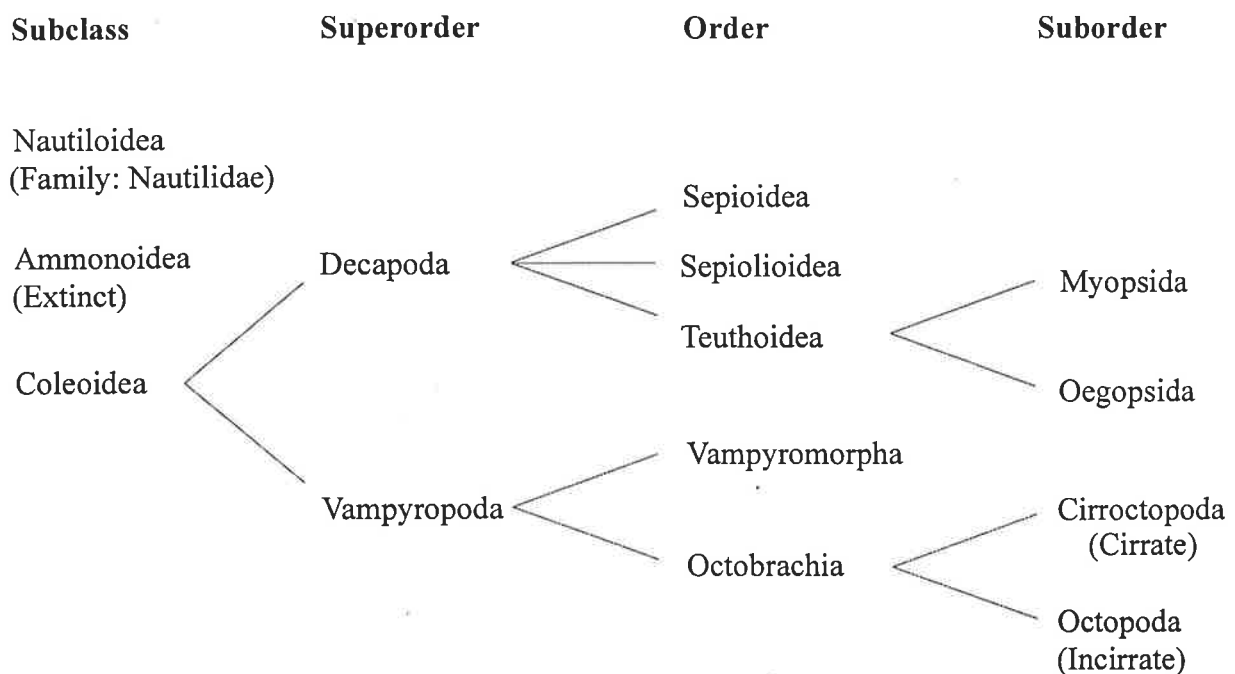
Figure 1.1: Cephalopod classification (from Boletzky 1998)

Cephalopods are dioecious, the males producing elaborate spermatophores, which are transferred to the female during copulation, prior to fertilisation of the eggs. Each egg is enveloped within a paired membrane as it exits the oviduct, and in most groups the eggs are further enclosed within a gelatinous membrane or matrix composed of a complex of mucosubstances produced by the oviducal and nidamental glands (Sugiura and Kimura 1995). The sperm must penetrate the egg coatings to fertilise the eggs, which are then released either separately or as a series of several to many eggs at one time. The production of eggs encased within a gelatinous coating is not restricted to the Cephalopoda. Indeed, this mode of reproduction for aquatic developing organisms has arisen independently in numerous phyla which is testimony to its success. Organisms producing eggs with a gelatinous coat include representatives from the anthozoans (Hand and Uhlinger 1991), nemerteans (Schmidt 1934), chaetognaths (Alvarino 1990), echinoderms (Thomas et al. 1999), gastropods (Pechenik 1979, Rawlings 1994, Chaparro et al. 1999), polychaetes (Hardege and Bartelshardege 1995, Sato and Osanai 1996, Lee and Strathmann 1998, Pernet 1998), insects (Rousch et al. 1997), amphibians (Seymour 1994, Marco and Blaustein 1998) and fish (Riehl and Patzner 1998).

The success of this mode of reproduction may partially result from the multiple purposes of the encapsulating material. One of the roles of the egg coating is as a sperm attractant (Ikeda et al. 1993, Sato and Osanai 1996), which is essential for fertilisation in some species (Olsen and Chandler 1998). Throughout development the egg coating provides protection from environmental extremes including desiccation (Fretter and Graham 1962, Pechenik 1978, Rawlings 1995), osmotic stress (Pechenik 1982, 1983, Woods and Desilets 1997), and exposure to solar radiation (Biermann et al. 1992, Rawlings 1996). The egg coating also presents a barrier in defence against predators (Spight 1977, Brenchley 1982, Rawlings 1990, Rawlings 1994) and pathogens (Lord 1986, Wourms 1987, Rawlings 1995). In some species the egg coating may provide a nutritive source for the developing embryo (Rivest

1986, Garin et al. 1996, Miloslavich 1996). The egg coating is also important in retaining the eggs at favourable sites (Strathmann 1995, Pechenik 1986, Rumrill 1990, Riehl and Patzner 1998). In addition, the incorporation of gelatinous material into egg masses has implications for gas exchange, by reducing the density of embryos and therefore competition for oxygen between siblings (Seymour and Bradford 1995, Lee and Strathmann 1998). The elastic nature of gelatinous egg coatings also enables capsules to swell, increasing the surface area for gas exchange (Seymour 1994).

Within the Mollusca, the diversity of designs of egg coatings incorporated with eggs is extreme, ranging from single to numerous eggs incorporated within gelatinous ribbons and masses or multi-layered capsules. Most of the eggs are characterised by small size, small ovum, short developmental time and hatching at larval or pre-metamorphosed stages. However, cephalopods produce comparatively large eggs that have a long developmental duration and direct development into juveniles that resemble miniature adults. Within the Cephalopods, egg or egg mass design is as diverse as the size of the eggs (Fig. 1.2 and 1.3), which range over three orders of magnitude from the eggs of *Todarodes pacificus* with an ovum volume of <0.5 mm^3, to the eggs of *Sepia apama* with an ovum volume exceeding 600 mm^3, and possibly even larger ovums in the cirrate octopods (Fig. 1.3d,e). Small eggs are typically contained within an amorphous gelatinous mass characteristic of the ommastrephid squids. Large eggs are usually individually encased within a series of spirally coiled jelly layers which form a tough capsule as is characteristic of *Sepia*, the sepiolid squids and the pygmy cuttlefish (Fig. 1.2f, g), or a rigid outer capsule as in the eggs of cirrate octopods. Some of the teuthoid squids deposit eggs in a similar type of spiralled jelly protection, but include several to many eggs per 'capsule' (Fig. 1.2a). Conversely, the jelly is much reduced or absent in the incirrate octopods, however the eggs are brooded and protected throughout the developmental period. The egg size provides some indication of the mode of development of the hatchling. A smaller egg typically has a short developmental duration

and produces a small planktonic juvenile, whereas a larger egg provisions a long development period and a larger benthic juvenile (Fioroni 1990).

Both ecological and physical factors impose limitations on the design of the egg or egg mass. Ultimately egg size will be limited by the size of the female, with large eggs produced by females exceeding 20 cm in mantle length. However, egg size does not necessarily correlate with maternal size and large eggs (10 mm) may be produced by small adults (45 mm mantle length) as is the case in *Rossia pacifica* (Anderson and Shimek 1994). The finite energy for egg production must be partitioned between the number and size of eggs. Energy allocated to egg production includes the investment in protective jelly, reducing that available for ovum production. Therefore the egg size will also depend on the way in which energy is distributed between the number of eggs and the design of the egg protection. If the success of the juvenile is increased by large hatchling size (Blaxter 1969, Hutchings 1991), selection of large hatchlings will favour large ovum size (Spight 1976). In addition to these ecological influences, physical factors will affect egg design. The embryos require oxygen from the environment for successful development (Adolph 1979). Oxygen must diffuse from the external environment through the egg capsule to the embryo. Diffusion through an aquatic medium is a slow process (Dejours 1988), which is exacerbated by thick capsules. The rate of oxygen consumption of embryos enclosed within capsules may be constrained by the morphology of the egg and the availability of ambient oxygen (Chaffee and Strathmann 1984, Seymour and Roberts 1991, Booth 1995, Strathmann and Strathmann 1995, Cohen and Strathmann 1996). In bird (Vleck and Vleck 1987), reptile (Vleck and Hoyt 1991) and amphibian eggs (Seymour and Bradford 1995), the oxygen consumption rate increases as egg size increases, therefore the likelihood of diffusion limited oxygen consumption increases with increased egg size. Thus diffusive limitation to oxygen consumption constrains both the size of individual eggs and egg masses.
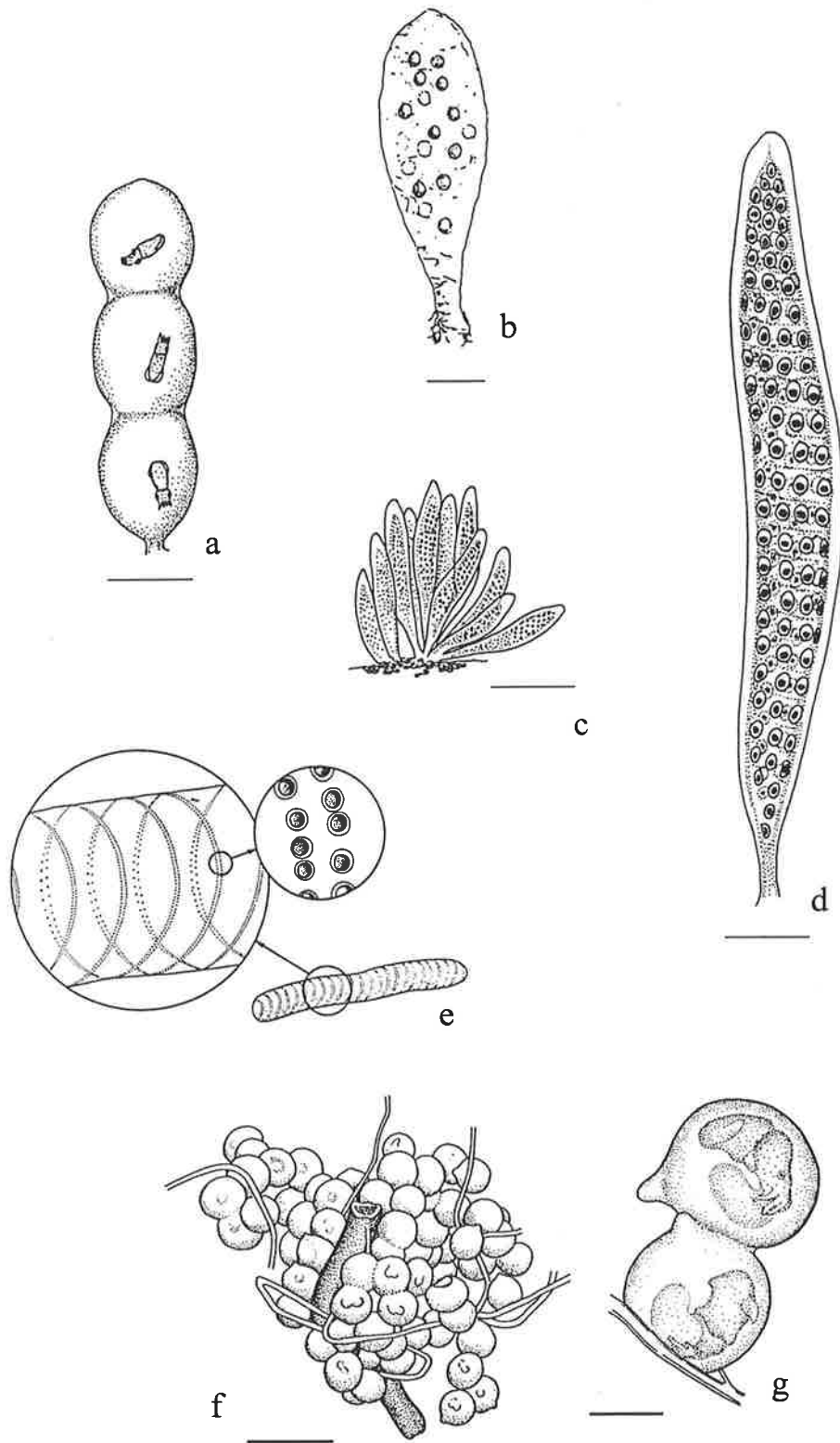
Figure 1.2: Decapod eggs and egg masses: a, *Sepioteuthis sepioidea* (scale bar = 13 mm) (from Roper 1965); b, *Alloteuthis* sp. (scale bar =10 mm) (from Jatta 1896); c, *Loligo plei* (scale bar = 36 mm); d, *Loligo plei* showing spiral arrangement of eggs within nidamental jelly (scale bar =9 mm) (c and d from Hanlon et al. 1992); e, *Thysanoteuthis rhombus* floating egg mass (100-130 cm length) showing detail of double helix egg string (Sabirov et al. 1987); f, *Sepietta oweniana* egg mass (scale bar = 1 cm); g, *Sepietta oweniana* eggs (scale bar = 2 mm) (Bergstrom and Summers 1983).
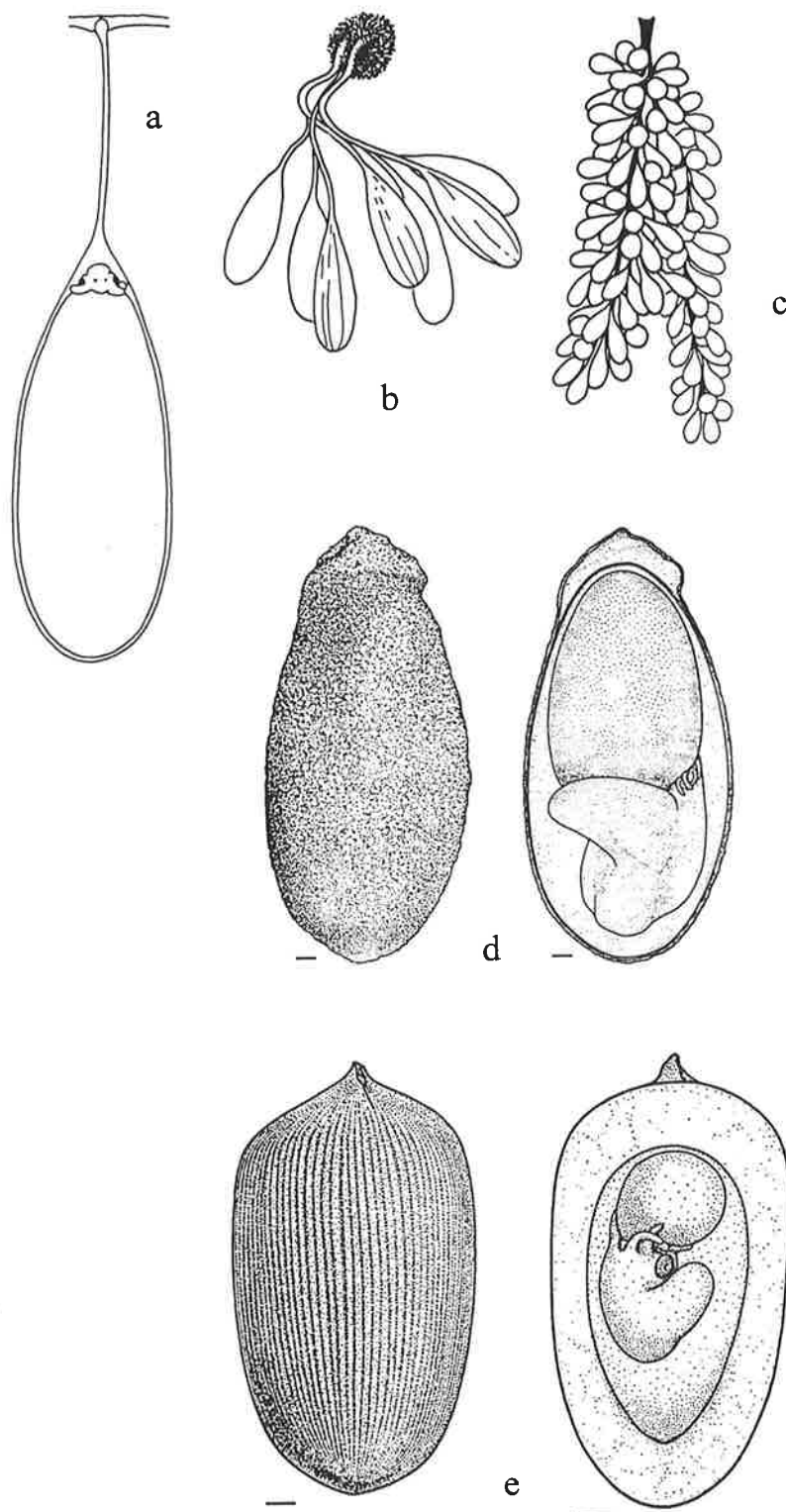
Figure 1.3: Vampyropoda eggs and egg masses: a, *Octopus conispadiceus* egg (capsule length = 18 mm) (from Ito 1983); b, *Eledone cirrhosa* egg cluster (capsule length = 7 mm) (from Fioroni 1978); c, *Octopus vulgaris* egg strings (capsule length = 2.5 mm) (from Fioroni 1978); d, Cirromorph egg showing outer capsule and exposed embryo with chorion approaching egg capsule size; e, Cirromorph egg showing outer capsule and exposed embryo with chorion membrane surrounded by gelatinous material (scale bars = 1 mm) (d and e from Boletzky 1982).

The eggs of *Sepia apama* are among the largest mollusc eggs deposited in coastal waters and are expected to represent the upper boundary for sufficient gas exchange. The lemon-shaped eggs (Fig. 1.4) are laid in mid-Autumn in southern Australian waters and left unattended throughout the 3-5 month developmental period, after which they hatch into juveniles. Each egg consists of a single yolk enclosed by a vitelline and chorion membrane surrounded a tough jelly-like capsule, which is attached to the roofs of caves or crevices by a gelatinous stalk (Fig. 1.5). Initially the vitelline membrane contacts the chorion membrane, however as development proceeds, water moves by osmosis through the chorion membrane, creating a fluid-filled space between the two membranes (DeLeersnyder and Lemaire 1972). This space is termed the perivitelline space (PVS), and the fluid within it the perivitelline fluid (PVF). The PVF surrounds the developing embryo, providing a buffer against physical stress. Further support is provided by layers of jelly surrounding the chorion membrane, and by a firmer capsule layer on the outermost surface of the egg. Eggs are deposited in dense clusters, which may include the eggs of several females from multiple matings laid at different times, as is the case in *Sepia officinalis* (Tinbergen 1939).

Figure 1.4: *Sepia apama* eggs deposited on the underside of a sheet of corrugated iron in 5 m depth at Edithburgh jetty.
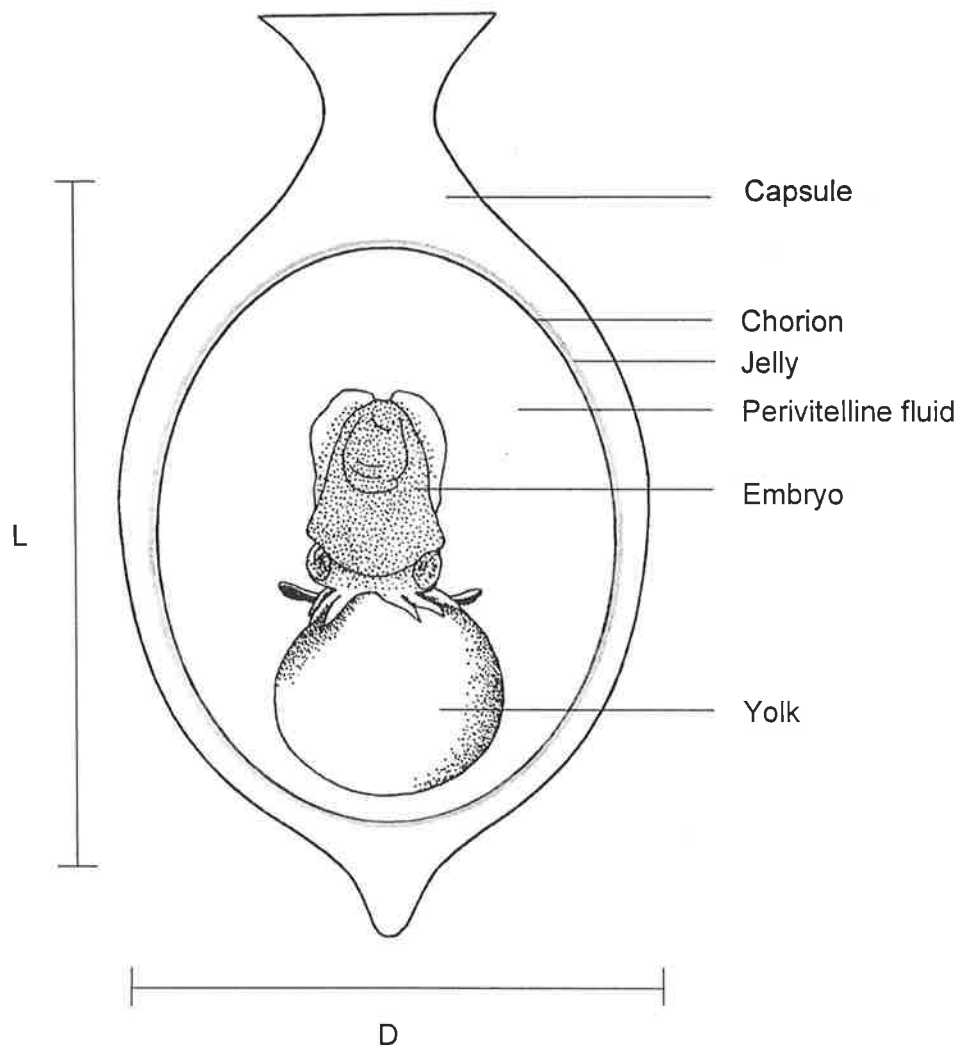
Figure 1.5: *Sepia apama* egg. The egg consists of a hardened outer jelly coat or capsule, soft jelly layer and chorion membrane, which encloses the perivitelline fluid, and developing embryo with attached yolk. The dimensions of the egg (length (L) and diameter (D)) change throughout the developmental period.

This study concerns morphological and physiological aspects of the eggs of *Sepia apama*. Changes in the morphology of the egg that are necessary to maintain a suitable environment for the growth of the embryo throughout development are documented in Chapter 2. Growth of the embryo involves a series of sequential morphological changes that are described in staging tables in Chapter 3. The development of the embryo and maintenance of the egg

micro-environment requires energy supplied by the oxidation of the yolk. The rate of oxygen uptake of the egg and embryo is quantified in Chapter 4, and the implications of oxygen limitation discussed. Chapter 5 concerns the energy expenditure involved with the development of an embryo from the yolk.

Morphology and growth of the egg

Introduction

Throughout development, the morphology of cephalopod eggs changes with the stage and metabolism of the embryo. Upon exposure to seawater, the gelatinous material expands rapidly and forms an outer leathery or rigid layer in individually laid eggs, but generally does not harden in egg masses (Boletzky 1998). The inner jelly of individually laid eggs then gradually diminishes as the perivitelline fluid (PVF) is formed. The PVF results from the accumulation of water between the vitelline and chorion membranes, termed the perivitelline space (PVS). The PVF increases throughout development, providing a buffer against mechanical injury and changes in ambient salinity (Pechenik 1983) or acidity (Taylor 1973). The formation of PVF causes varying degrees of capsule expansion, depending on the degree of initial hardening of the jelly. Expansion increases the capsule surface area and reduces the capsule thickness, which respectively increases the area for oxygen uptake and decreases the diffusive distance for oxygen, facilitating gas exchange (Wolf *et al.* 1985, Chapter 4). This is needed to support the increasing metabolic demands of the developing embryo. A thinner capsule also presents a less formidable barrier when hatching.

Morphological changes occurring in the egg are dependent on the development of the embryo. Embryonic development involves an increase in mass and differentiation of tissues, the pattern of which has interested investigators for centuries. The pattern of growth has been extensively studied in birds (Stark and Ricklefs 1998), and has extended more recently to reptiles (Deeming and Ferguson 1991), amphibians (Hota 1994), and fish (Rombough 1988a, Finn 1994). However, little work has focussed on the patterns of embryonic growth of molluscs (Bayne 1983) and specifically of cephalopods, other than the examination of the effects of temperature on developmental period and the construction of staging tables (Chapter 3), although a few studies have investigated changes in the morphology of

cephalopod eggs throughout development (Jecklin 1934, Boletzky 1987b). The apparent lack of information for cephalopods is surprising since the eggs of some species are relatively easy to obtain in large quantities, which is necessary for destructive sampling throughout development to monitor growth. Embryonic growth has historically been classified as either sigmoidal or exponential following work on bird eggs. Sigmoidal growth is characterised by an initial slow growth phase, followed by a rapid expansion phase, and finally by a slow plateau phase nearing hatching. Species exhibiting a sigmoidal growth pattern typically have precocial young that are largely independent of parental care. Exponential growth does not slow near to hatching, and the altricial young produced are fully dependent on parental care. Various mathematical models to characterise embryonic growth have been developed, which enable comparisons within and among species to assess the variability of growth modes (Ricklefs 1987, Ricklefs and Starck 1998).

The present chapter describes and quantifies the morphological changes occurring in *Sepia apama* eggs throughout development, which are fundamental for the normal development of the embryo. The changes in mass of the components of the egg and of the embryo are quantified throughout development, and the embryonic growth pattern is described.

Methods

Site collection and egg maintenance

S. apama eggs were collected in May-August from Edithburgh jetty, Yorke Penninsula, South Australia and from the Broken Hill Proprietary (BHP) metal works slag heap located at Whyalla, Eyre Penninsula, South Australia (Fig. 2.1). Field collection sites were chosen for their proximity to Adelaide, the accessibility for diving and the protection from weather extremes. Dives were made fortnightly from mid-April in 1995-1999 to ensure that the beginning of the egg laying season was identified and that collection was as soon after laying as possible.
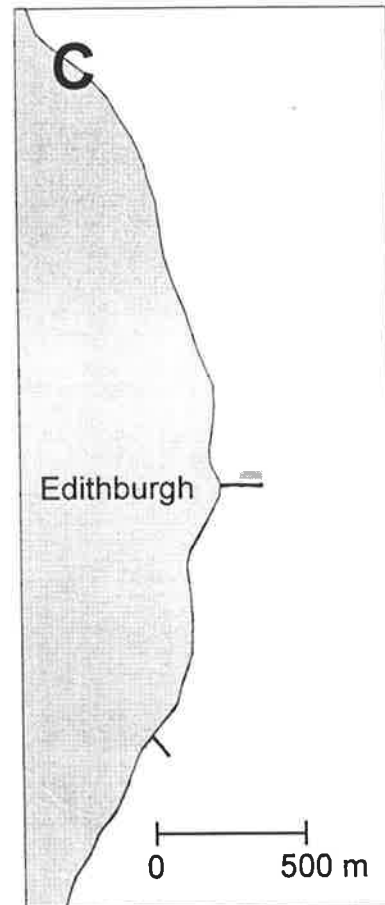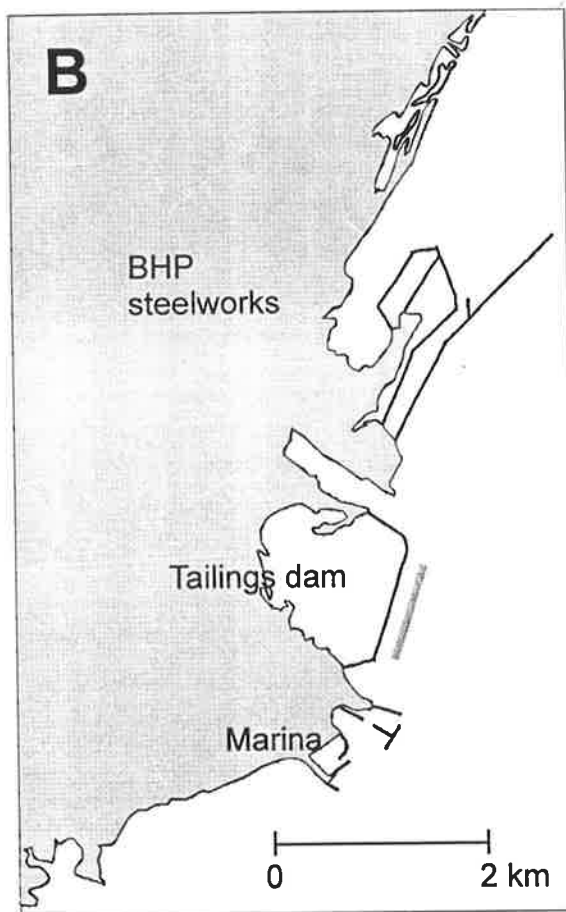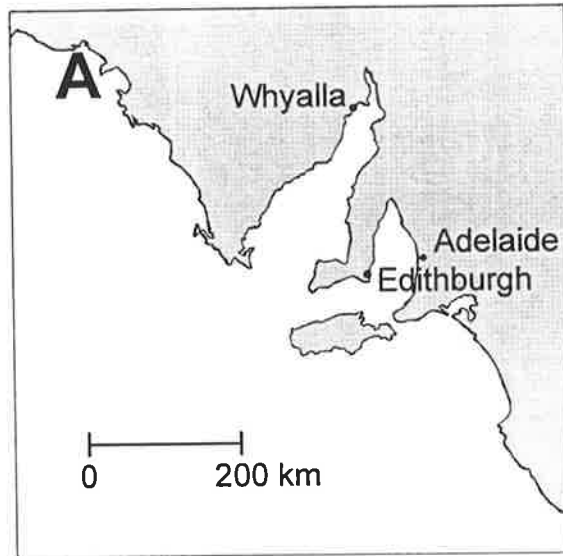
Figure 2.1: A, Southern South Australia, showing collection sites relative to Adelaide; B, Egg collection site (grey line) along tailings dam wall, BHP steelworks, Whyalla; C, Egg collection site (grey line) north of Edithburgh jetty.

The Edithburgh jetty protrudes east from the coast and has a dredged channel along the northern side. As a result of dredging, there is a limestone reef running parallel to and approximately 25 m distant from the jetty. The reef is approximately 50 m in length and occurs at the eastern end of the jetty. Another smaller reef protrudes approximately 20 m from the end of the jetty. In both reefs there are numerous holes and narrow caves, varying in size from 30-50 cm in width, 10-30 cm in height and 60-80 cm deep (Rowling 1994). *S. apama* eggs were laid deep within these dens, which made egg collection difficult. Between the reef and the jetty two artificial dens were introduced following the collection of eggs in the initial season (1996) from underneath a sheet of corrugated iron. The artificial dens consisted of two sheets of corrugated iron held apart by a 5cm wide piece of wood nailed at each of the short ends. Eggs were not found in these artificial dens until 1998. Due to the unreliability and difficulty in obtaining eggs from Edithburgh, a second site at Whyalla was used in 1997-1999.

The Whyalla BHP metal works slag heap consists of slag piled into the sea, approximately 30 m wide and extending several hundred metres into the bay. The wall facing out to sea attracts hundreds of cuttlefish that compete for mates and egg laying sites among the rock wall from March onwards. Removing and upturning rocks revealed numerous eggs on the undersurface of rocks, especially on larger flattened rocks at 3-5 m depth. A 'group' was defined as a collection of more than 100 eggs on an individual rock. Groups of eggs were carefully prised from rocks using fingertips and placed into plastic bags underwater. Neither the number of clutches nor the time of deposition of clutches within a group of eggs could be determined at collection.

Eggs were transferred to an insulated box filled with seawater, where they were kept for up to two days prior to being placed in sand-filtered flow-through seawater tanks, maintained at 12°C at the South Australian Research and Development Institute (SARDI) Aquatic Sciences Centre, West Beach, South Australia. Individual eggs were threaded with a 10 cm

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length of cotton loop and suspended from a 5 × 5 cm polystyrene float, to mimic their orientation in nature.

Ageing eggs

The time of laying could not be determined and so the exact age of the eggs was not known. Therefore a method to estimate the age of the eggs was developed by labelling each group of eggs and measuring the dorsal mantle lengths (DML) of embryos at regular time intervals. By arbitrarily assigning day 0 as the time of collection, DML was plotted against time, which resulted in a series of curves representing 'clutches' within the groups of eggs (Fig. 2.2a). Each clutch was then shifted laterally to overlap with one another to form one continuous trend (Fig. 2.2b). In a similar manner, groups of eggs were shifted laterally to overlap with one another, enabling an estimate of the relative age of all eggs from the DML. A least squares, 3rd order polynomial was fitted to the data, generating a smooth curve that provided an age (day) for each egg (DML) measured (Fig. 2.2c). An independent check of the aging method was possible by measuring the DML of a clutch of eggs of known age (collected immediately after laying and placed in flow-through sea water aquaria maintained at 12°C). At 80 days old, the DML was 4 mm which corresponded to the estimate of 87 days from grouping the eggs according to their DML. Subsequent data were plotted against estimated age of the eggs.

Morphology of the eggs and embryos

Twelve eggs from each group were selected every three weeks from SARDI and transferred to aquaria in a constant temperature room at 12°C at the University of Adelaide, South Australia. Eggs were removed from the aquaria, placed on absorbent paper, and weighed. Egg length (the longest distance excluding the stalk and the tail) and diameter were measured using calipers (Fig. 1.4). Once the egg became too large to maintain its shape out
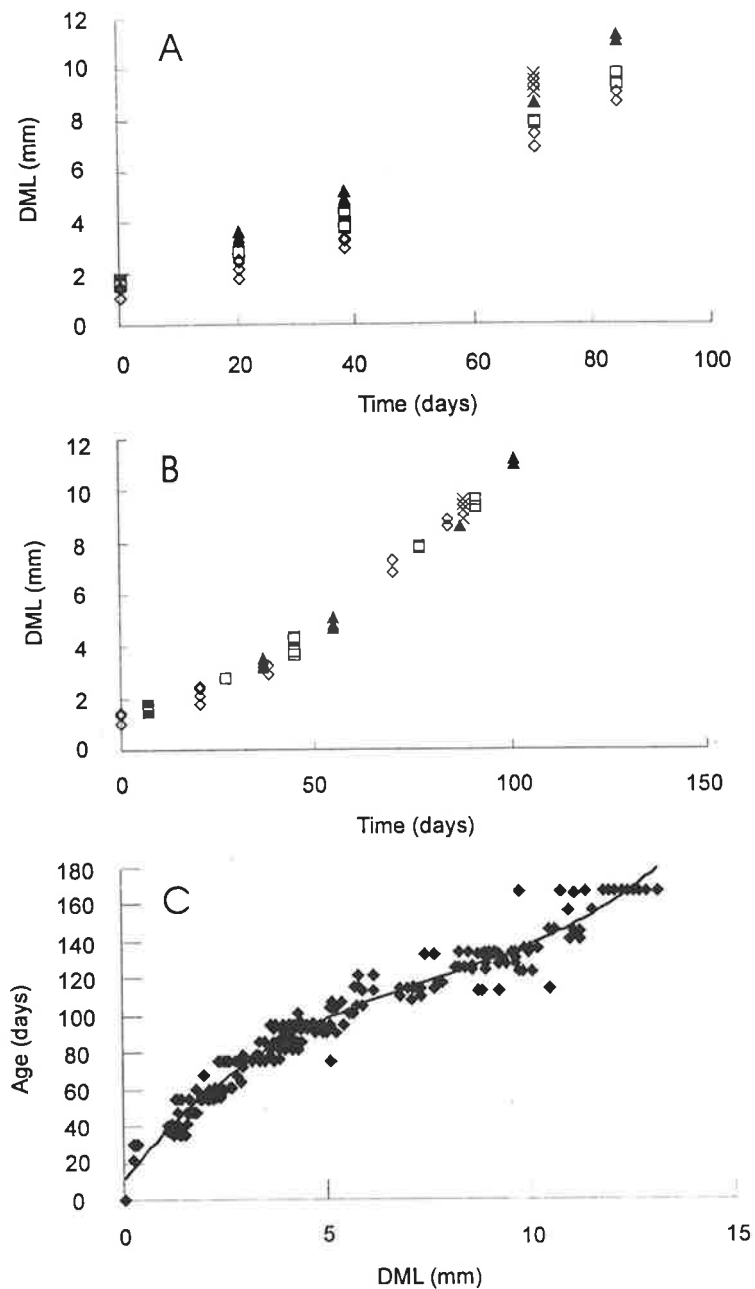
Figure 2.2: Relationship between dorsal mantle lengths (DML) and time for a) a single group of eggs with b) clutches shifted laterally to coincide (symbols represent different clutches within a group). All groups c) were combined to estimate the age of the embryos as a function of their DML (each point represents an individual embryo (n=296)). The 3rd order polynomial used to describe the data is; age = $0.12 \times DML^3 - 2.78 \times DML^2 + 27.96 \times DML + 11.27$, $r^2 = 0.97$.

of water, the egg dimensions were taken whilst suspended in water. Eggs were sliced longitudinally with a razor blade through the capsule to remove the chorion containing the perivitelline fluid (PVF), yolk and embryo. The thickness of the capsule (X) was measured at six places along the longitudinal cut with a dissecting microscope and ocular micrometer.

The effective surface area (ESA) of each egg was calculated assuming the egg to approximate the shape of a prolate spheroid. The ESA was determined from the geometric mean of the semi-axes (A and B) whereby $A = (A_o A_i)^{0.5}$, and $B = (B_o B_i)^{0.5}$, with A_o = egg length/2 and B_o = egg diameter/2 and subscripts o and i denoting the outside and inside radii respectively:

$$ESA = 2\pi B^2 + (2\pi AB)/e \cdot \sin^{-1} e$$

$$\text{where } e = \text{eccentricity} = (A^2-B^2)^{1/2}/A$$

The embryo with attached yolk (Y_{ext}) was placed in autoclaved seawater and the dorsal mantle width (DMW) and the dorsal mantle length (DML) were measured using a dissecting microscope and ocular micrometer. A subset of embryos representative of the entire developmental period was fixed in 1.25% gluteraldehyde. The embryos were rinsed with phosphate buffered saline, blotted and weighed to the nearest 0.1 g. The embryos were sliced in half longitudinally through the ventral mantle and the internal yolk (Y_{in}) flushed out using fresh water. Yolk-free embryos (E_{yf}) were blotted and weighed, then frozen and dried overnight in a Dynavac FD-5 freeze drier prior to weighing to determine the mass of E_{yf} throughout development, which enabled calculation of mass-dependent metabolic rate (Chapter 4).

Perivitelline fluid

The PVF was collected by inserting an 18-gauge needle through the chorion into the perivitelline space and drawing all of the fluid into a 1 or 2.5 ml syringe and measuring the

volume to the nearest 0.1 ml. The PVF was placed into 5 ml vials and frozen for osmolality analysis. The osmolality of the PVF was determined using a Wescor 5100c vapour pressure osmometer. Samples (8 μL) were processed following calibration with 290 and 1000 mmol kg^{-1} Wescor osmolality standards.

Growth rate

Throughout development a subset of eggs was blotted and the masses measured on a balance. Eggs were individually frozen in liquid nitrogen for 45 s. As each egg gradually thawed, the capsule was sliced around the circumference and each capsule half removed. The capsule mass was measured as above, and the thickness measured at six equidistant positions around the circumference of the cut as above. Removing the capsule exposed the PVF enclosing the embryo and yolk. The PVF was carefully chipped into a weighing tray, exposing the embryo and yolk. The embryo was separated from the yolk once it was sufficiently large and the mass of the embryo and yolk measured separately. Component parts of the egg (capsule, embryo and yolk) were prepared for dry mass measurements as above.

Variations in egg size

Each season a subset of young eggs (with no embryo observable and no PVF) were selected representing a range of egg sizes. Eggs were dissected and prepared as above. In addition, eggs collected in 1999 were grouped according to egg size (large or small) and maintained at 15°C in aquaria until hatch. At three intervals during development six eggs were randomly selected from each size group and the mass and dimensions of the egg measured, prior to dissection to measure PVF volume, capsule thickness and embryo DML. Approaching hatch, the aquaria were regularly monitored to check for hatchlings, which were removed, blotted and weighed and DML measured. Hatchlings were fixed as above, prior to

dissection to remove the Y_{in}. The wet and dry mass of the hatchling and the Y_{in} mass were measured.

Data analysis

To summarise the data, the eggs were grouped into age categories from 0-40 days, then every 20 days to hatch. The numbers of replicates within each age category vary because the age of the eggs could not be accurately determined when measured. Data are presented as mean ± standard error, followed by the number of replicates.

Results

Field observations

The water temperature in the field reached a minimum of during July, then gradually increased in the following months (Fig. 2.3). Eggs were predominantly found on the underside of flattened rock or other substrate at 3-5 m depth in dense clusters sometimes two layers thick. When horizontal substrate became limiting, as at the slag heap wall, eggs were deposited in crevices on any available rock face or even on exposed rocks. The capsule was initially soft when freshly laid, but rapidly hardened upon exposure to seawater. Newly laid eggs were white and opaque, but throughout development the eggs swelled and gradually became translucent, enabling observation of the embryo within the capsule. Late in development the eggs had expanded such that adjacent eggs contacted and pressed against one another (Fig. 2.4). Eggs that became pressed together were coated in a layer of fine particles, suggesting water velocity past the egg clusters was slow. This is in contrast to the eggs at earlier developmental stages, which were rarely fouled. The exclusion of light may be important in minimising fouling, as the eggs on the periphery of a cluster near a rock edge were often coated by pink coloured algae and several eggs laid in exposed positions were heavily fouled by algae. Predation on the eggs was not directly observed, however at
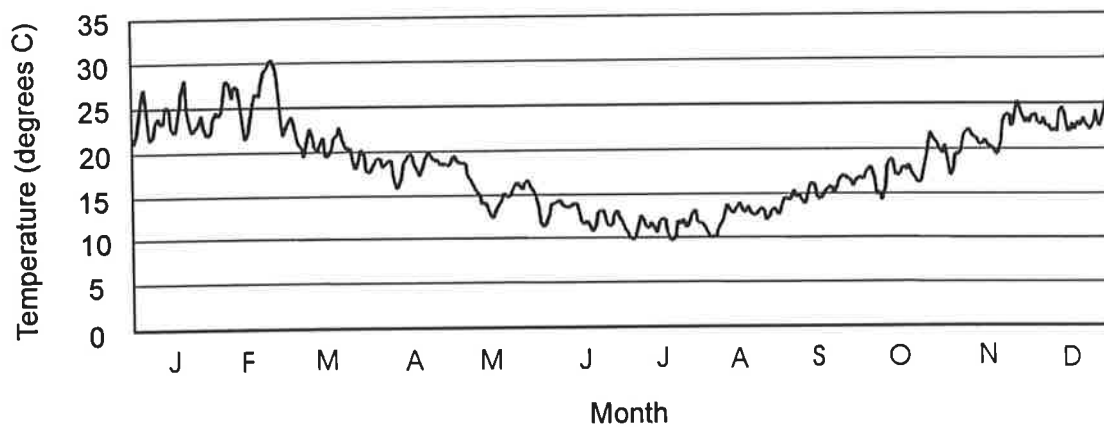
Figure 2.3: Average water temperature in coastal waters of Spencer Gulf (Warburto Point) during 1997. Data courtesy of Seddon (in prep).
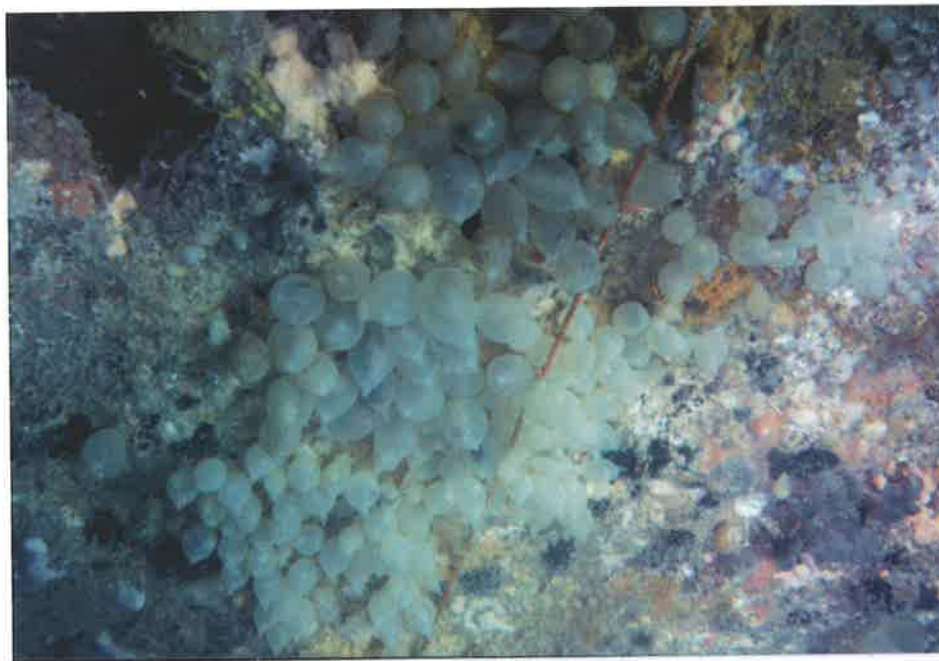
Figure 2.4: *Sepia apama* eggs clustered together on the underside of a rock at Whyalla. Several clutches are identifiable by the vastly different sizes of the eggs. The increase in volume of the PVF throughout development results in late stage eggs pressing together. Note also on the right hand side of the figure the egg stubs resulting from removal by sea urchin predators (photo credit: Val Boxall).

Whyalla, the sea urchin, *Heliocidaris erythrogramma*, was regularly removed from egg clusters with large patches of egg stubs remaining. At Edithburgh, pygmy leatherjackets, *Brachaluteres jacksonianus,* have been observed attacking and consuming eggs (Bavendam 1994). When a rock was overturned late in development, the eggs were disturbed and many of the embryos hatched. The hatchlings swam down between the eggs, then underneath nearby rocks.

Egg morphology

'Young' eggs were white in colour, weighed 2.13 ± 0.05 (6) g and approximated 12.8 ± 0.01 (12) by 17.7 ± 0.02 (12) mm (Fig. 2.5 and 2.6). As the embryo developed, the egg expanded, became more translucent, and often discoloured. At 12°C the incubation period was approximately 160 days. Prior to hatching the egg mass increased to 4.07 ± 0.03 (12) g and measured 1.76 ± 0.02 (12) by 2.35 ± 0.08 (12) cm. Expansion of the egg occurred by absorption of water across the capsule and chorion into the perivitelline space. PVF volume increased from 0.54 ± 0.17 (9) to 2.93 ± 0.35 (10) ml in the last 100 days of development (Fig. 2.7). The expansion of the egg stretched the capsule, thinning the wall from 1.52 ± 0.07 (7) to 0.43 ± 0.09 (11) mm while ESA increased from 6.54 ± 0.14 (12) to 11.73 ± 0.74 (11) cm^2 (Fig. 2.8). Measurements of mantle dimensions were not possible until 35-40 days when the mantle of the embryo had developed sufficiently. The width of the mantle exceeded the length until approximately day 110, when the relationship reversed. At hatching, the DML was 12.01 ± 0.21 (16) mm.
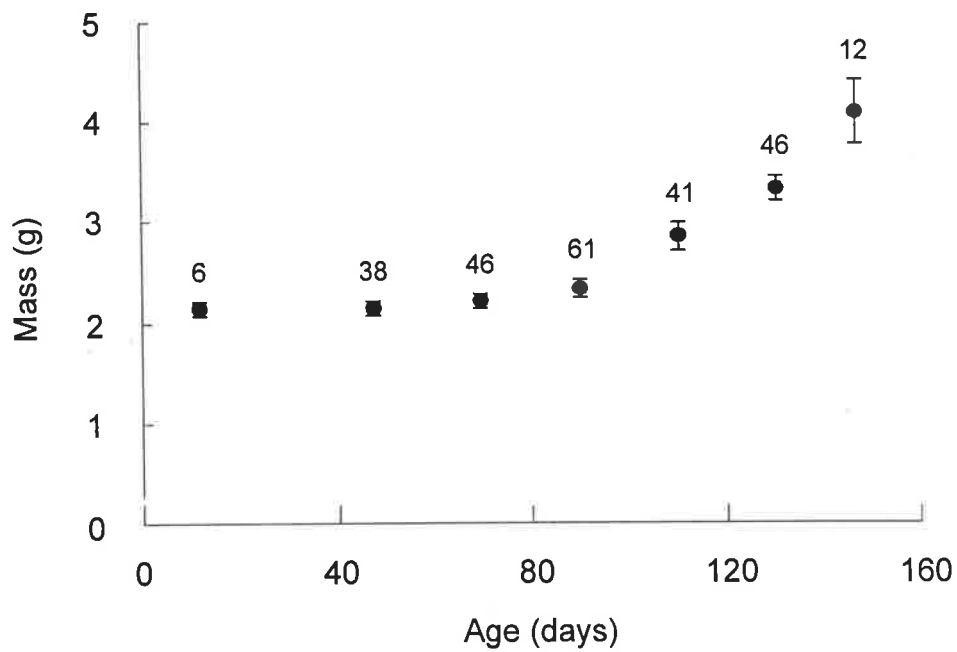
Figure 2.5: Changes in egg mass throughout development at 12°C. The numbers of replicates in each age category are indicated above the points. Bars represent standard error of the mean.
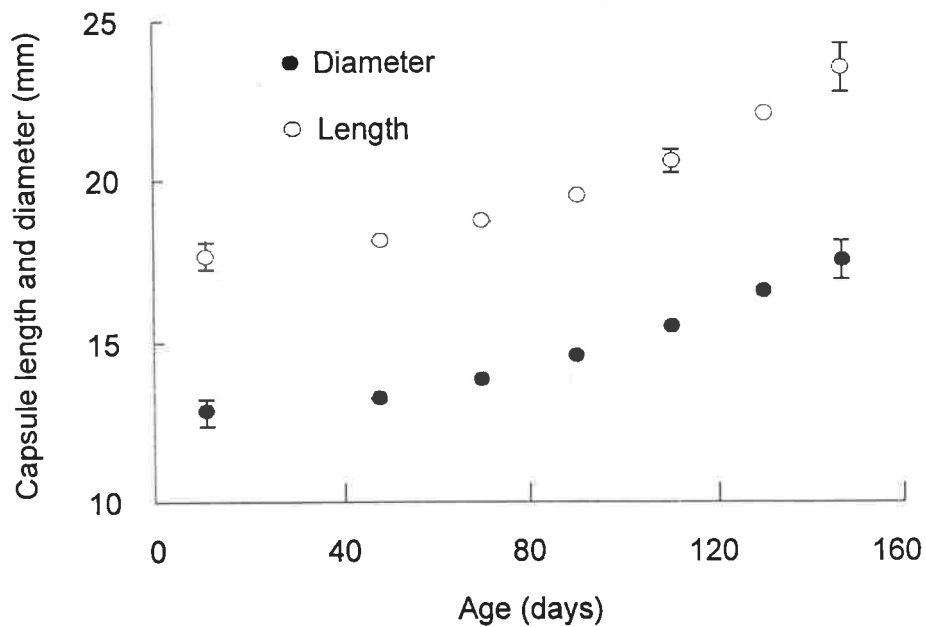
Figure 2.6: Changes in egg dimensions throughout development at 12°C. The number of replicates in each age category varied between 12 and 61. Bars represent standard error of the mean.
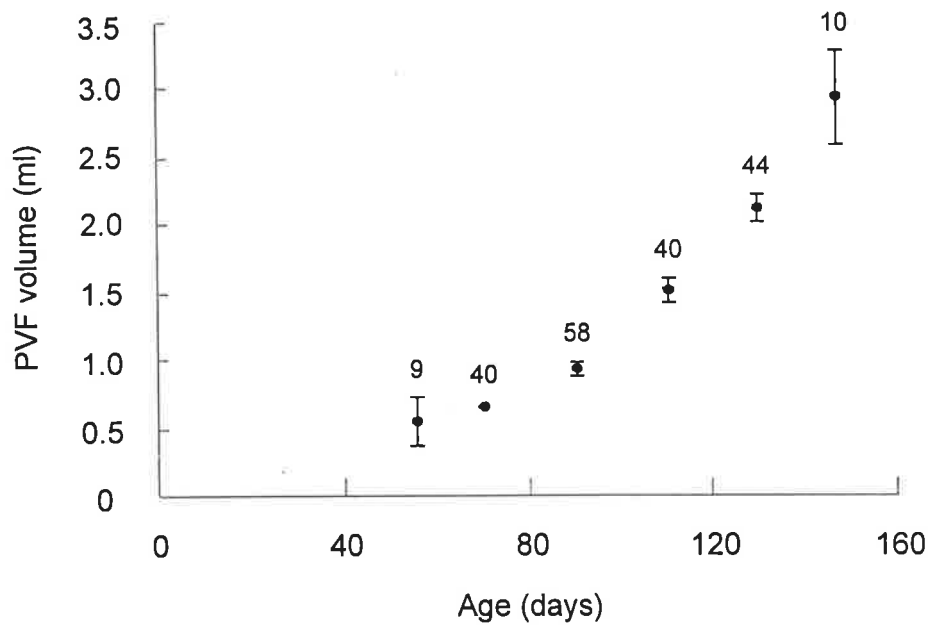
Figure 2.7: Changes in perivitelline fluid volume throughout development at 12°C. The number of replicates in each age category are indicated above the points. Bars represent standard error of the mean.
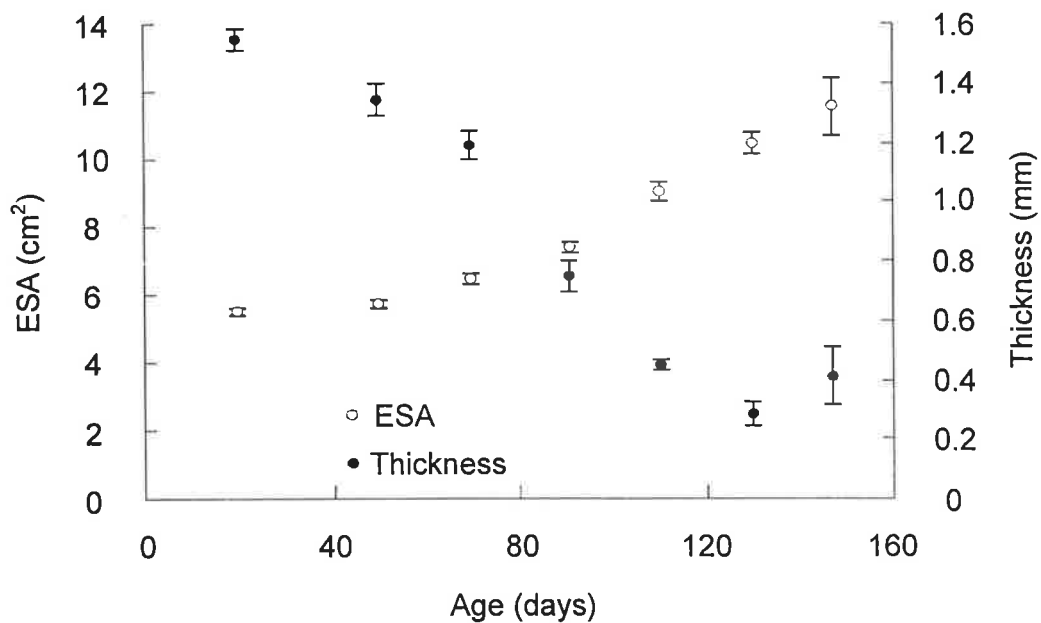
Figure 2.8: Changes in effective surface area (ESA) and thickness of the capsule throughout development at 12°C. The number of replicates in each age category varied between 10 and 61. Bars represent standard error of the mean.

Perivitelline fluid osmolality

The total osmolality of the PVF decreased by 125 mmol kg^{-1} during the final 70% of development, but was always greater than the osmolality of the seawater. At 50 days the difference between the PVF and seawater osmolality was 275 mmol kg^{-1}, but this reduced to 150 mmol kg^{-1} by 150 days.
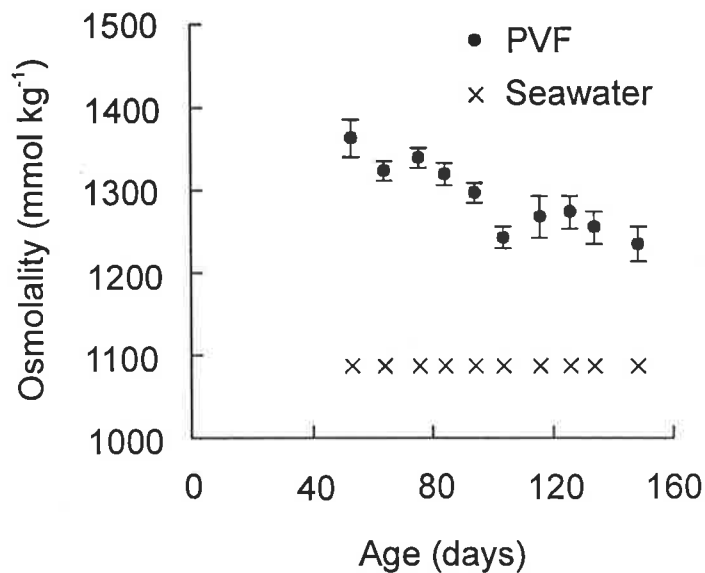
Figure 2.9: Changes in osmolality of the perivitelline fluid (circles) and seawater (crosses) throughout development at 12°C. The number of replicates in each age category varied between 7 and 17. Bars represent standard error of the mean.

Growth rates

Increases in the total egg mass reflected the increase in volume of the PVF, however changes in egg mass also resulted from a gradual decrease in both capsule and yolk wet mass, and increase in embryo wet mass (Fig. 2.10). The mass of the capsule included the jelly layers immediately beneath the capsule, which gradually diminished as the PVF volume increased.

In addition to the diminishing mass of the jelly, layers from the outside of the capsule were gradually sloughed off, resulting in a decrease in the capsule mass from 1.770 ± 0.059 (17) g at the beginning of development to 0.742 ± 0.086 (7) near to hatching. The capsule dry mass showed a similar trend decreasing from 0.148 ± 0.003 (16) to 0.075 ± 0.006 (7) g (Fig. 2.11). Comparing the wet and dry capsule masses shows that the capsule constitutes 90% water.

The wet mass of the yolk showed very little change with time, decreasing in only the final 30 days of development to reach 231.0 ± 24.5 (7) mg prior to hatch. The dry mass of the yolk was approximately ½ of the wet mass, decreasing to 103.5 ± 11.5 (7) mg (Fig. 2.12).
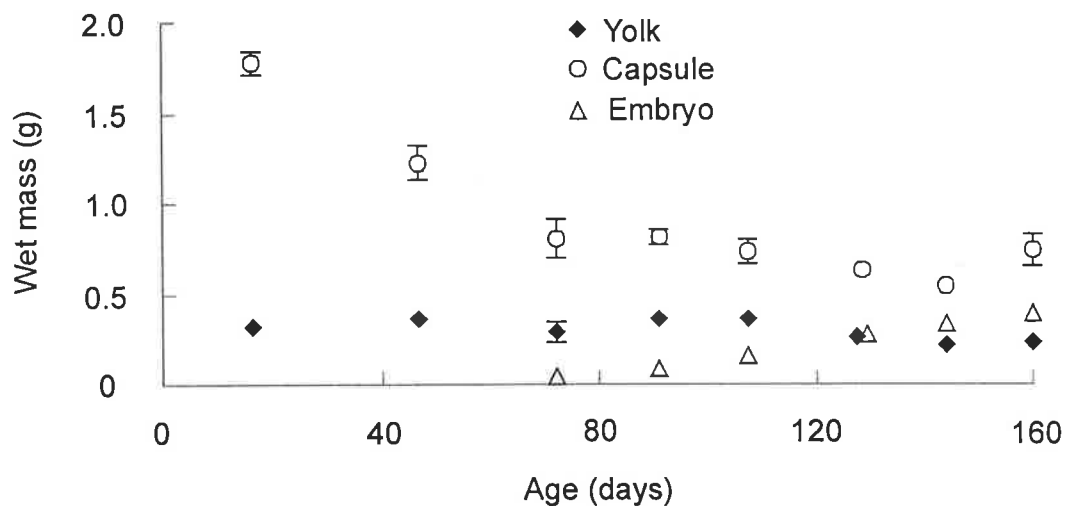
Figure 2.10: Changes in wet mass of the egg components throughout development at 12°C. The number of replicates in each age category varied between 6 and 28. Bars represent standard error of the mean.
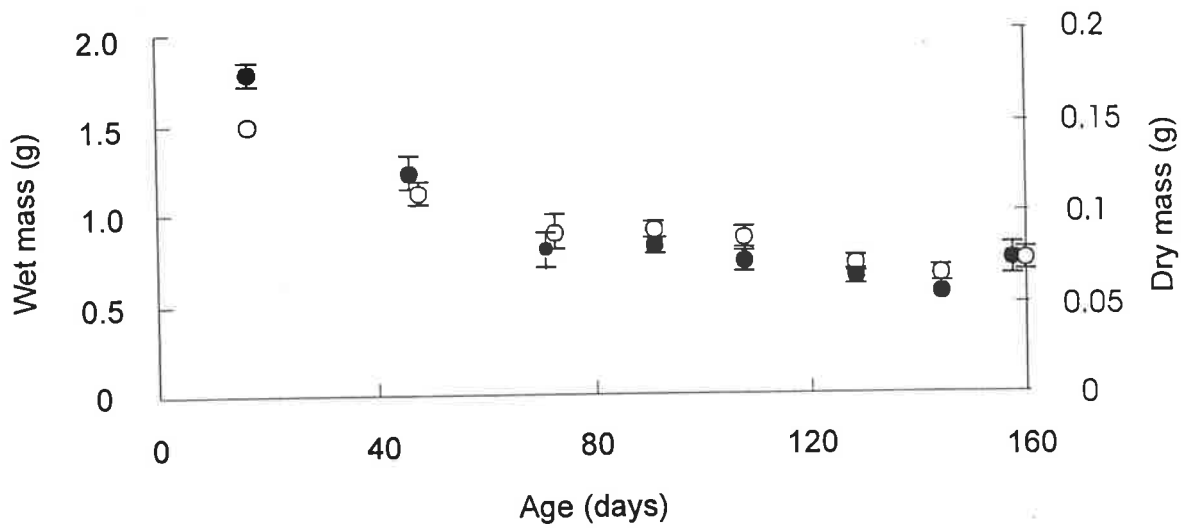
Figure 2.11: Changes in mass of the capsule throughout development at 12°C, wet mass (filled) and dry mass (open). The number of replicates in each age category varied between 7 and 28. Bars represent standard error of the mean.
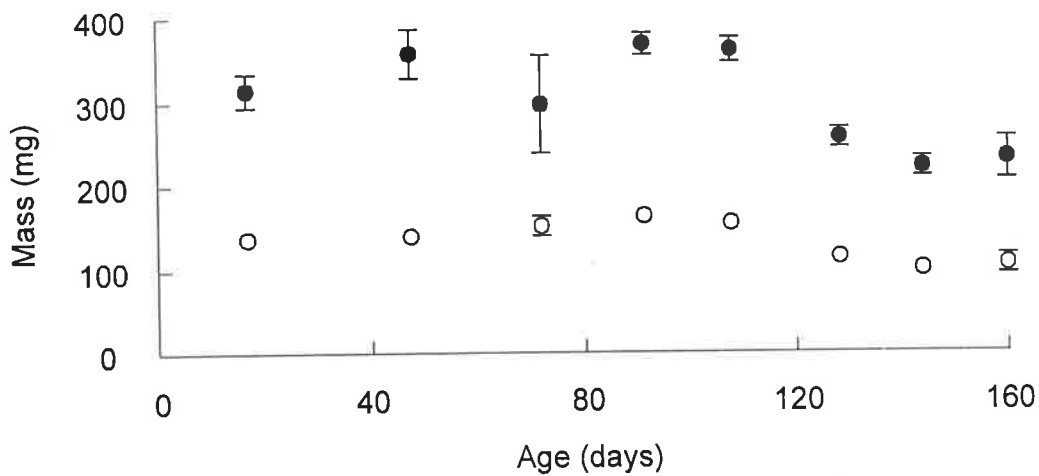
Figure 2.12: Changes in mass of the yolk throughout development at 12°C, wet mass (filled) and dry mass (open). The number of replicates in each age category varied between 6 and 27. Bars represent standard error of the mean.

The embryo first became apparent to the naked eye viewed through the egg capsule as a clover shaped bud on top of the yolk, which rapidly grew to resemble a miniature adult. During development, some of the external yolk sac was transferred into internal yolk sacs within the mantle. Embryo wet mass including the internal yolk (E) increased exponentially from 16.6 ± 3.2 (8) mg at 48 days to reach 345.5 ± 20.8 (23) mg at 149 days (Fig. 2.13). Hatchlings weighed 496.2 ± 9.5 (20) mg. Embryo dry mass reflected wet mass, increasing from 3.0 ± 0.6 (8) mg to 78.9 ± 3.6 (21) mg over the same period, with hatchlings of 102.3 ± 4.2 (20) mg. The internal yolk-free embryo mass (E_{yf}) was measured directly following dissection. The E_{yf} mass at 134 days was 28.0 ± 1.7 (3) mg, increasing to 52.2 ± 1.5 (7) g for hatchlings (Fig. 2.14). Removal of the internal yolk (Y_{in}) was destructive, therefore the dry mass was estimated using the proportion of dry mass contained in the external yolk (0.45) multiplied by the difference between E and E_{yf}. With an initial mass of 0.8 ± 0.5 (8) mg the Y_{in} made up 26% of the total embryo mass. The proportion of Y_{in} increased throughout development to a maximum of 48% at 134 days. The Y_{in} of hatchlings was slightly lower (44%) however, some consumption of the yolk may have occurred between the time of hatching and dissection (Fig. 2.14).
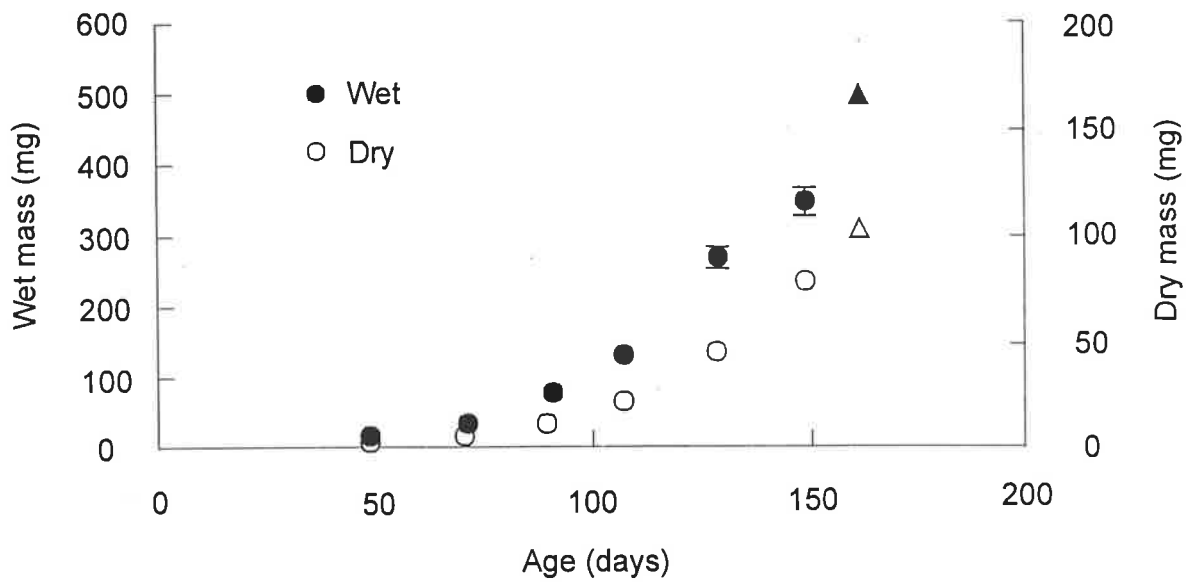
Figure 2.13: Changes in embryo wet and dry mass throughout development at 12°C. Triangles represent hatchlings. The number of replicates in each age category varied between 8 and 36. Bars represent standard error of the mean.
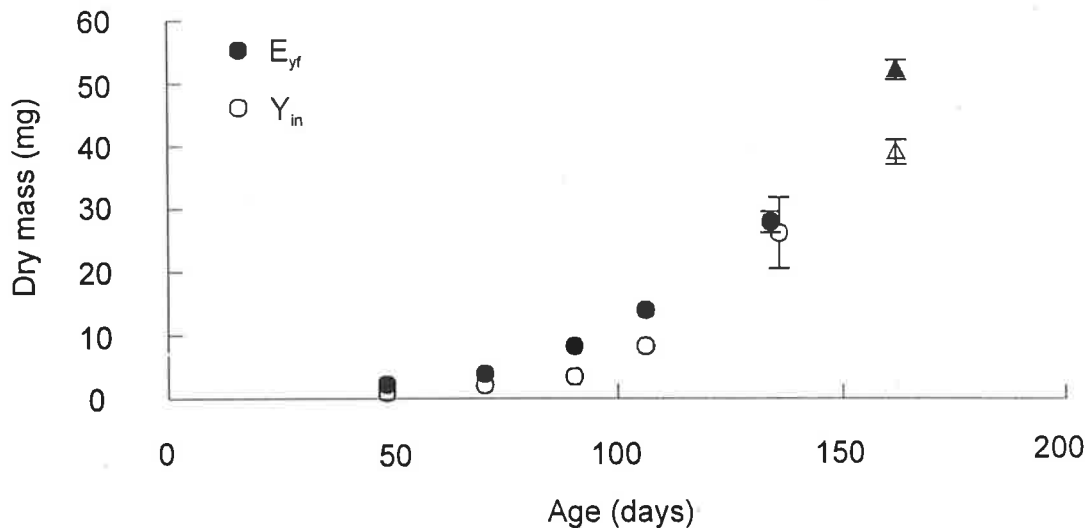
Figure 2.14: Changes in internal yolk free embryo (E_{yf}) mass and internal yolk (Y_{in}) mass throughout development at 12°C. Triangles represent hatchlings. The number of replicates in each age category varied between 7 and 18. Bars represent standard error of the mean. The exponential equation for the E_{yf} mass = $0.0006 \times e^{0.0282} \times$ age, $r^2 = 0.99$.

Eggs collected from the two sites during the study period averaged 4.4 ± 0.1 (315) g but ranged from 1.5 g to 9.9 g for eggs with no PVF or embryo apparent to the naked eye. The egg at 9.9 g was unusually large and contained two embryos - the only double-embryo egg encountered during the study.

Egg dry mass correlated positively with yolk, capsule and jelly dry mass (Fig. 2.15). The relationship between capsule mass and egg mass can be described by a power equation whereby; capsule dry mass = 0.57 × egg dry mass$^{1.26}$ (r^2 = 0.86). The proportion of the structural components of the egg (capsule and jelly) increased disproportionately as egg size increased, whereas the proportion of the yolk component of the egg decreased with increased egg size (Fig. 2.16). The greater capsule mass of large eggs also provided thicker capsules (r^2 = 0.67) (Fig. 2.17).
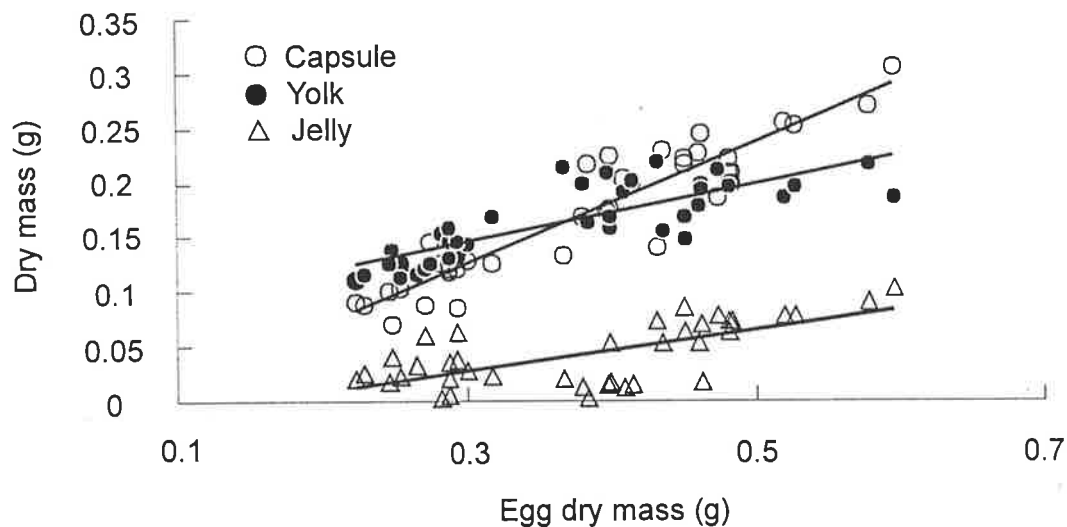
Figure 2.15: Changes in the dry mass of egg components of variable initial egg mass. The linear equations describing the data are: capsule mass = 0.5481 × egg dry mass - 0.0373, r^2 = 0.88 (n = 40); yolk mass = 0.2633 × egg dry mass + 0.0667, r^2 = 0.66 (n = 40); jelly = 0.1828 × egg dry mass - 0.0267, r^2= 0.46 (n = 39).
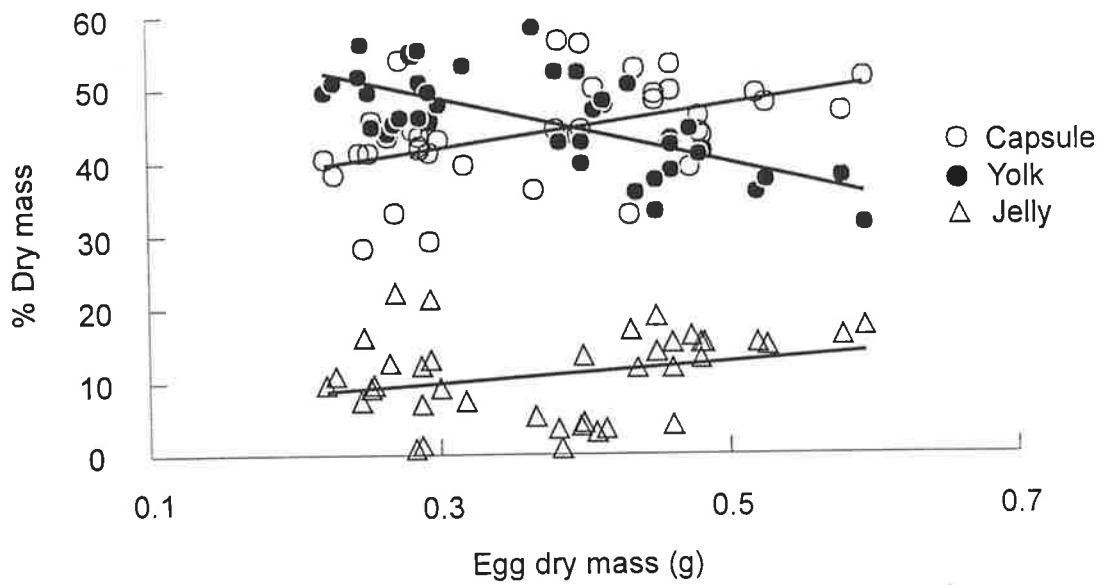
Figure 2.16: Egg component dry mass as a proportion of initial total egg mass. The linear equations describing the data are: capsule mass = $28.56 \times$ egg dry mass + 33.36, $r^2 = 0.20$ (n = 40); yolk mass = $-44.92 \times$ egg dry mass + 62.22, $r^2 = 0.49$ (n = 40); jelly = $14.07 \times$ egg dry mass + 5.51, $r^2 = 0.06$ (n = 39).
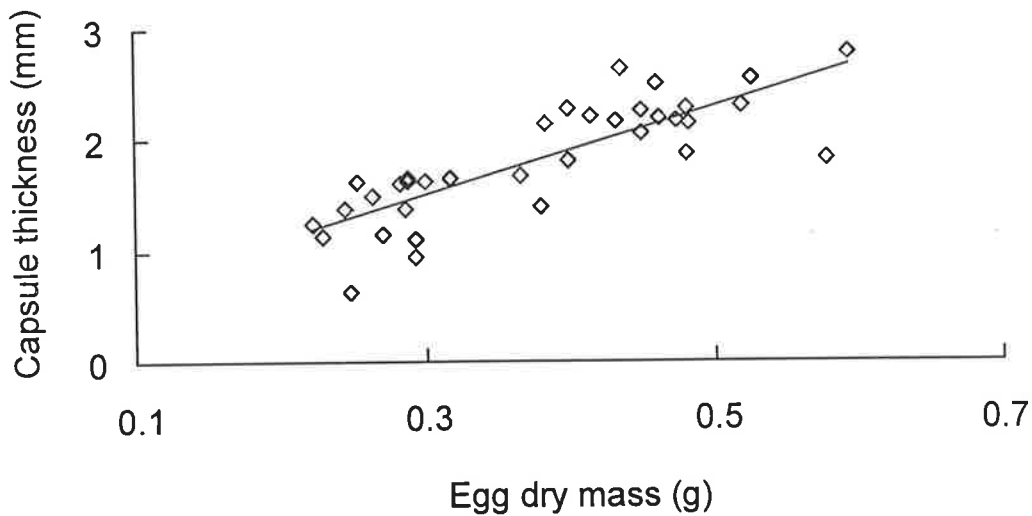
Figure 2.17: Changes in the capsule thickness of initial egg mass. The linear equation describing the data is: capsule thickness = $3.9861 \times$ egg dry mass + 0.3033, $r^2 = 0.67$ (n = 35).

Eggs incubated until hatch were grouped into two categories, large and small which were significantly different in mass ($p < 0.001$) and effective surface area ($p < 0.001$). A significant difference between large and small egg mass and ESA remained at least until 91 days of development (Fig. 2.18a and b). At day 55 and 91, the PVF volume of large eggs appeared to be greater than the small, however no significant difference was detected (Fig. 2.19a). Similarly, despite a trend of greater capsule thickness for large eggs at day 23 and 55 (Fig. 2.17b), no significant difference was detected. The DML could not be measured until day 55, however at 55 and 91 days there was no significant difference between embryos from large compared to small eggs. Likewise, the DML of hatchlings was not significantly different (Fig. 2.20), however the cuttlebone of hatchlings from large eggs was longer (13.19 $\pm$ 0.13 (9) mm) than hatchlings from small eggs (12.37 $\pm$ 0.34 (6)) ($p < 0.05$) (Fig. 2.20). Wet mass measurements of the whole and dissected hatchlings from large eggs were significantly greater than hatchlings from small eggs, however the dry hatchling mass, % water, and wet and dry internal yolk masses were not significantly different (Fig. 2.20).
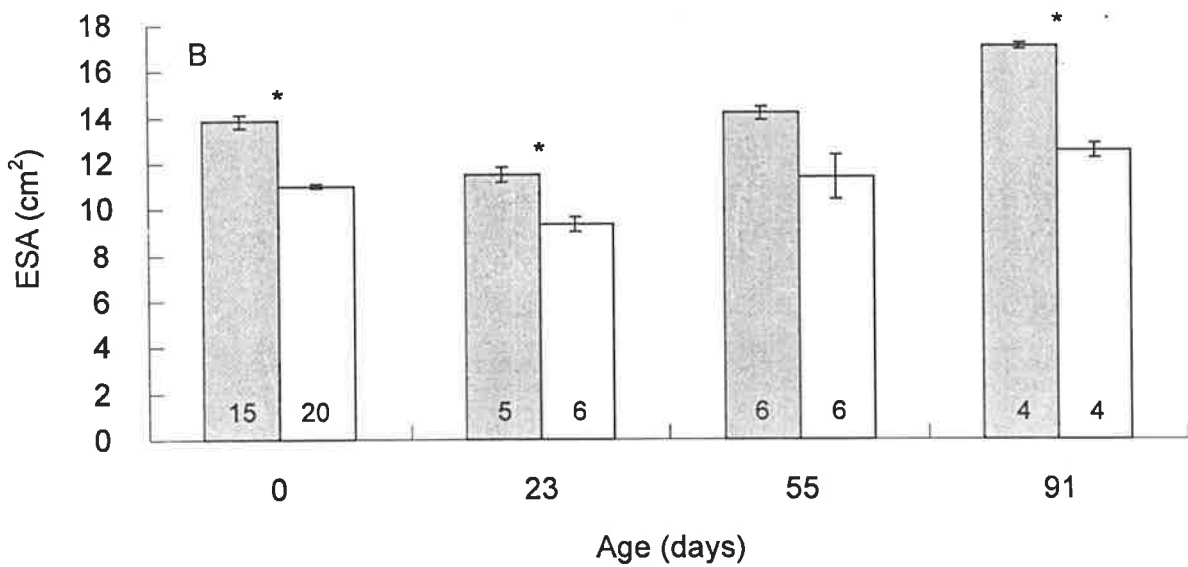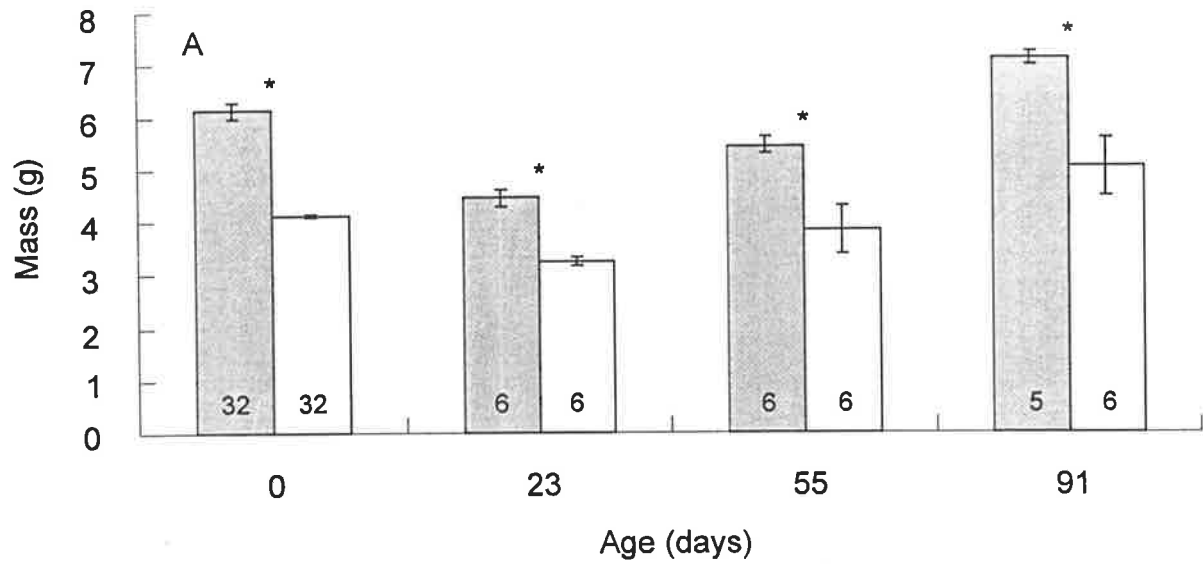
Figure 2.18: Comparisons between large (grey) and small (white), egg mass (A) and effective surface area (B), during development at 15°C. The number of replicates in each age is indicated at the base of the columns. Significance (p<0.05) is indicated by *. Bars represent standard error of the mean.
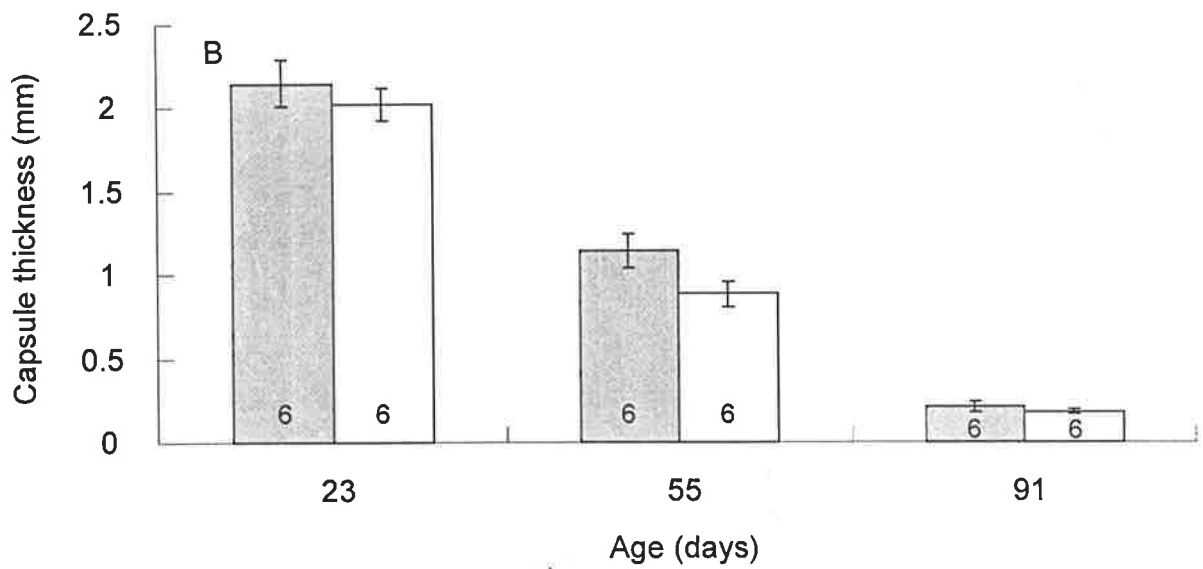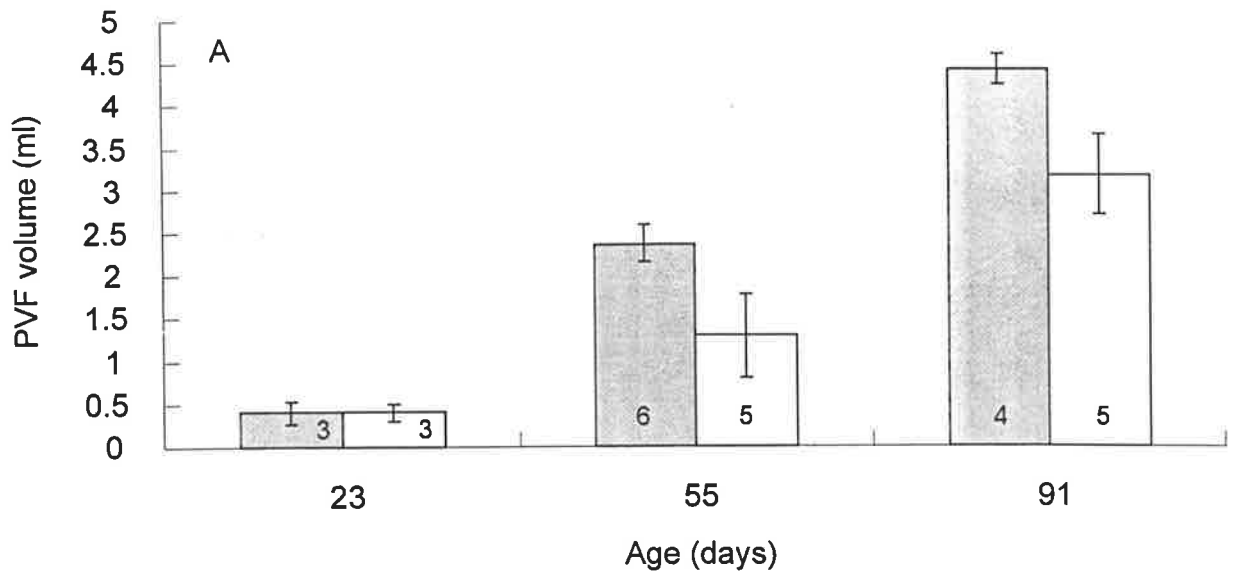
Figure 2.19: Comparisons between large (grey) and small (white), perivitelline fluid (PVF) volume (A), and capsule thickness (B) during development at 15°C. The number of replicates in each age is indicated at the base of the columns. Bars represent standard error of the mean.
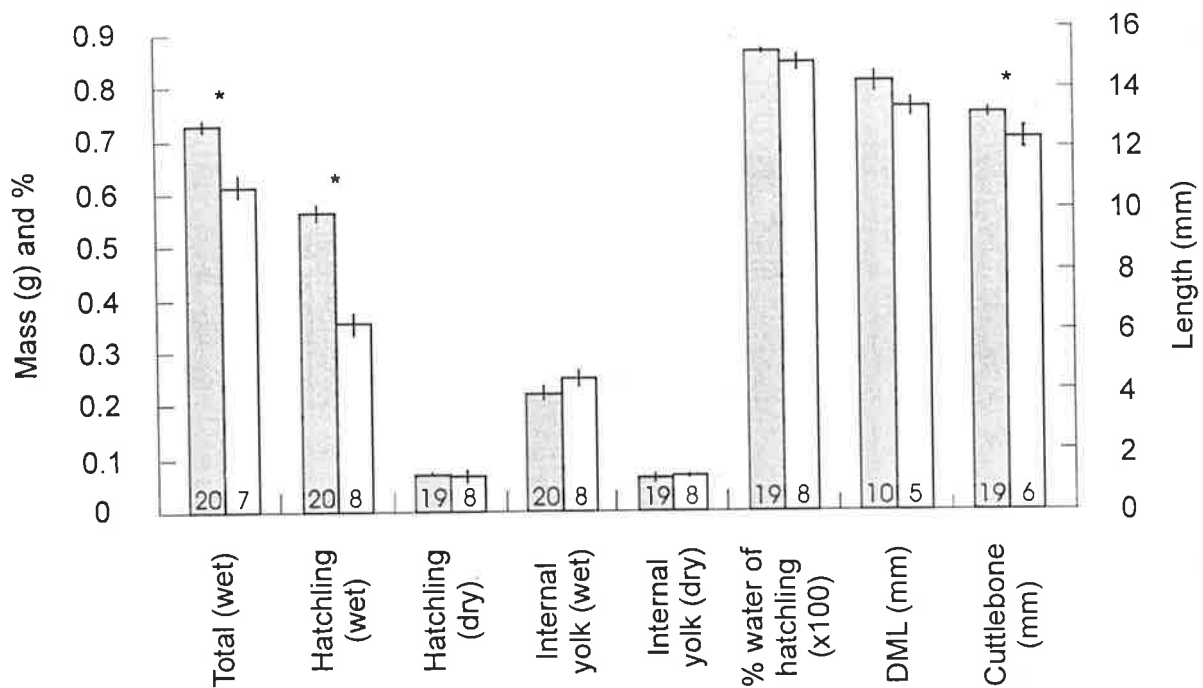
Figure 2.20: Comparison between hatchlings from large (grey) and small (white) eggs incubated at 15°C. Note the right hand axis relates only to dorsal mantle and cuttlebone length. The number of replicates is indicated at the base of the columns. Significance (p<0.05) is indicated by *. Bars represent standard error of the mean. For comparisons by Anova, average values from pairs of small eggs were used to obtain similar values for the number of replicates as the large eggs.

Discussion

Morphology

Morphological changes of *S. apama* eggs are initially gradual. There are slight increases in capsule dimensions, effective surface area (ESA), thickness and egg mass during the initial 60-70% of development. These morphological characteristics change more rapidly in the latter 30-40% of development (Fig. 2.5, 2.6 and 2.8), with the expansion of the egg as a result of water uptake across the semi-permeable chorion membrane into the hypertonic perivitelline fluid (PVF) (Fig. 2.7). The increase in PVF volume primarily accounts for the

increased egg mass and results in a 180% increase in total egg volume over the developmental period. Approaching hatch, the egg may have expanded so much that the capsule can split, exposing the translucent chorion and distorting the shape of the egg. This frequently happened in the lab, probably as a result of handling, but was also observed in the field. Increases in capsule volume are necessary to accommodate the final dimensions and increased activity of the hatchling, however, the increase in volume is more than enough to accommodate the embryo suggesting the large increase in PVF volume has an alternate function involving increased surface area for gas exchange (Chapter 4). Increases of between 80 – 150% in cephalopod eggs have been recorded, with the degree of expansion roughly correlating to the ovum size (Wells and Wells 1977). Jecklin (1934) found that the amount of PVF per gram of embryo was constant between two species which deposited egg masses, despite the large difference in egg size (egg size of *Loligo vulgaris* = 2 x *Alloteuthis subulata*). However, the relative water content of eggs incorporated in masses was less than that of the individually encased eggs of *S. oweniana* and *S. officinalis* (Jecklin 1934). Like *S. apama*, the individually encased eggs are much larger than the eggs incorporated in egg masses, necessitating a larger PVF volume to facilitate the metabolic needs of the large embryo (Chapter 4).

Perivitelline fluid

The osmolality difference between the PVF and seawater of 150 mmol kg^{-1} in *S. apama* eggs is similar to the value of 135 mmol kg^{-1} determined between the surrounding medium and the PVF of the eggs of the cuttlefish *Sepiella japonica* (Gomi *et al.* 1986). In *S. japonica* eggs, swelling of the egg results from a constant osmotic pressure caused by a linear increase in the protein content of the PVF (Gomi *et al.* 1986). The protein concentration is maintained at a constant level due to the accumulation of protein molecules, which are too large to permeate the capsule. Similarly, the intracapsular fluid of the gastropod, *Nucella lapillus,* contains large organic molecules which cannot diffuse through the capsule,

maintaining the osmotic concentration above that of the surrounding medium (Pechenik *et al.* 1984). The eggs swell due to the high permeability of the capsule to both water and inorganic ions (Pechenik 1983). In *S. apama* the maintenance throughout development of a higher PVF osmotic concentration compared to seawater results in a hydrostatic pressure inside the egg (Fig. 2.9). Swelling of *S. apama* eggs is more pronounced during the latter 30-40% of development (Fig. 2.7). Similarly, the increase in mass of *Sepietta oweniana* and *Sepia officinalis* eggs occurs mainly in the second half of development, whereas in *Loligo vulgaris* and *Alloteuthis subulata* eggs, the mass increases mainly during the beginning of development (Jecklin 1934). The latter two species have many eggs incorporated within jelly forming an egg mass, as opposed to the individually encased eggs of *S. apama*, *S. oweniana* and *S. officinalis*. A rapid early increase in the volume of egg masses may be necessary to reduce the embryo density sufficiently to enable adequate oxygenation, whereas individually encased eggs may have adequate exposure to oxygen early in development, but require increases in volume later in development as metabolic demands increase (Chapter 4).

Whilst the release of organic substances from the yolk or embryo which are too big to permeate the capsule wall is well established in many species' eggs (Bogucki 1930, Clegg 1964, Salthe 1965, Potts and Rudy 1969, Hall and MacDonald 1975, Peterson and Martin-Robichaud 1987, Alderdice 1988), the control of the release of these substances and the subsequent expansion of the eggs remains unclear. Previous studies on frogs eggs have suggested that the release of solutes is governed by the embryo, with the rate of expansion of *Rana pipiens* eggs determined by the developmental stage of the embryo (Salthe 1965). Similarly, Seymour *et al.* (1991) found that capsule conductance was most highly correlated to the embryonic stage of *Pseudophyrne bibronii* embryos further supporting the suggestion that the embryo releases solutes at specific developmental stages. In certain cephalopods, PVF formation is dependent on the presence of oviducal jelly in the gelatinous capsule (Ikeda *et al.* 1993, Ikeda and Shimazaki 1995, Sakurai *et al.* 1995, Sakai and Brunetti 1997),

suggesting the jelly plays a fundamental role in PVF formation early in development. Even the partial removal of the capsule resulted in the cessation of PVF formation in *Loligo vulgaris* (Jecklin 1934). Embryos without oviducal jelly began to develop, but became constrained by the chorion membrane and died. Mortality may have resulted from insufficient oxygenation of the egg owing to the reduced gas exchange of the smaller capsule (Chapter 4). Conversely, formation of the PVF in the octopods occurs independently of the presence of any gelatinous coating, indicating the jelly is only necessary for PVF formation in certain species.

Egg size

Variations between egg size at laying most probably reflect variations in maternal size within a species (Rawlings 1990, Ito 1997, Chaparro *et al.* 1999). Eggs collected at the beginning of the season were generally larger than those later in the season, which correlated with a greater frequency of encounters of large females early, as opposed to late in the season, at two sites in the vicinity of Whyalla (Hall and McGlennon 1998). However, some large eggs were also collected in late August suggesting that either some large females remained or small females are capable of producing large eggs.

Initial egg size also had significant consequences throughout development, most apparent in egg morphology. Initial differences in mass and ESA between large and small egg sizes remained, such that the mass of small eggs was approximately 70% of large egg mass at deposition and approaching hatch. Surprisingly, no significant difference in the PVF volume was determined despite the apparently greater volume in large eggs (Fig 2.19a), however this is likely to result from the small sample size, since PVF volume changed in direct proportion with egg mass during development (Fig. 2.5 and 2.7). Similarly, the small sample sizes for capsule thickness resulted in the trend of thinner capsules for small eggs being insignificant. The significantly smaller ESA and trend of thinner capsules for smaller eggs has

considerable implications for gas exchange (Chapter 4). Low sample sizes are also likely to account for the high variability in determinations of hatchling wet and dry mass, since dry mass and relative water content of the embryo were not significantly different, but measurements of hatchling wet mass were significantly different. If wet masses are different, and relative water content are not, clearly errors in sampling (probably during dissection) have occurred. Nevertheless, the longer cuttlebone and greater wet mass of hatchlings from large eggs indicates that larger eggs do indeed result in larger hatchlings.

Capsule

The increase in capsule dry mass scales disproportionably with increased egg mass with the exponent equal to 1.26 (Fig. 2.15). This value is similar to the average exponent of 1.12 describing the relationship between supportive tissue mass and body mass in a range of animals as diverse as spiders to whales (Anderson *et al.* 1979). The capsule of *S. apama* represents 28-56% of the total egg mass at the beginning of development in comparison to 4-17% of shelled terrestrial eggs (Anderson *et al.* 1979). However, the predominant function of the capsule of *S. apama* eggs is probably not structural, since water provides buoyancy and support, and similarly sized eggs of octopods are enclosed solely by the chorion. *S. apama* eggs swell causing the capsule to stretch and become thinner. The outer layers of the capsule are sloughed off, resulting in a 50% reduction in dry mass throughout development (Fig. 2.11). The greater capsule volume of *S. apama* eggs enables the extreme changes in egg morphology. The capsule also has a protective role, with thicker capsules potentially providing greater protection from predators or pathogens. Thick-walled capsules of various species of marine gastropod eggs are more resistant to puncturing, and predation by isopods, than are thin-walled capsules (Perron 1981, Rawlings 1990). However, for larger predators such as crabs, thicker capsules do not confer greater protection (Rawlings 1994). Predation on *S. apama* eggs by the sea urchin, *Heliocidaris erythrogramma*, was likely to be indiscriminate, whereas predation by pygmy leatherjackets, *Brachaluteres jacksonianus*,

which nibble at the egg case, may be influenced by the thickness of the capsule. Whether the larger, thicker-walled *S. apama* eggs provide more protection than smaller, thinner-walled eggs was investigated by offering eggs of different wall thickness to sea urchin and pygmy leatherjacket predators in the lab. No predation occurred on any of the eggs suggesting conditions in the lab are unsuitable and field studies are necessary to detect any benefits of thick capsules. The thickness of the capsule has also been proposed to correlate positively with the period of incubation in nudibranch and gastropod molluscs (Gibson *et al.* 1970, Spight 1975, Todd 1979). An extended incubation period exposes the eggs to potential risks for longer, necessitating greater investment in protective materials. This was found to be the case among marine gastropod *Conus* species, where the proportion of energy devoted to capsule material increased with developmental time and egg size (Perron 1981). Owing to their large size and long developmental duration, *S. apama* eggs are expected to have a high proportion of the total egg mass devoted to the capsule. The negative consequence of a thicker capsule is a greater energetic cost for the female (Chapter 5). The major role of the capsule is to protect the egg from bacterial attack (Chapter 4), and to enable morphological changes throughout development that are necessary for gas exchange.

Yolk

Yolk mass also increases disproportionately with increased egg mass, but represents a smaller proportion of total egg mass as egg mass increases (Fig. 2.16). *S. apama* has an initial dry yolk mass of 165.7 mg, which is more than double the dry mass of *S. officinalis* yolk (77.8 mg) (Bouchaud 1991), reflecting the difference in total egg wet mass between the two species (3.23 g (this study) compared to 1.31 g (Bouchaud and Galois 1990)). During development the external yolk diminishes as a result of metabolism to provide energy for embryonic growth, and transferral into paired internal yolk sacs (Chapter 3). The external yolk can be an indicator of premature hatching if yolk remains when the embryo hatches (Boletzky 1987a). The internal yolk represented almost 50% of the dry mass of the late stage

S. apama embryos, in comparison to 60% in *S. officinalis* (Bouchaud 1991). In *S. apama* the wet mass of the internal yolk represents 30% of the total hatchling wet mass, which compares with 14-15% in precocial birds, 8-10% in altricial birds, 2-28% in turtles, 1-12% in lizards, 11% in a species of snake and 5-30% in a species of crocodile (Vleck and Hoyt 1991). Residual yolk is necessary for hatchlings with high post-hatching energy requirements, such as digging from a nest (turtles, crocodiles and megapode birds), and would provide a limited energy source if food was scarce or difficult to obtain. The large proportion of yolk contained in *S. apama* could substitute for feeding if food was scarce (Chapter 5).

Embryo

Determinations of growth rate were dependent on measurements of DML, which were possible only after approximately 35-40 days at 12°C, when the mantle of the embryo had developed sufficiently (Fig. 2.2c). Embryo mass increased exponentially (Fig. 2.14) culminating in the production of a juvenile. The absence of a larval period and metamorphosis is typical of invertebrate embryos developing from large yolky eggs (Vance 1973, Christiansen and Fenchel 1979, Jaeckle, 1995, and Chapter 5). In avian terms the direct developing hatchling is characteristic of precocial development, whereby the hatchling is largely independent of parental care. However, precocial avian development is characterised by a sigmoidal pattern of growth with an initial slow growth phase, followed by a rapid expansion phase, and finally by a slow plateau phase nearing hatching. The mass of *S. apama* embryos did not plateau prior to hatch, indicating exponential growth. Indeed, there is no apparent decline in growth rate with increased size in cephalopods, making the application of asymptotic equations inappropriate (Forsythe and Heukelem 1987). Exponential growth is characteristic of altricial development in birds with hatching occurring earlier and hatchlings requiring extensive parental care. Clearly, *S. apama* does not follow either of these avian growth models and further analysis is necessary.

Numerous models describing the increase in embryo mass over time have been developed including polynomials, exponentials and sigmoidal equations, however either the complexity of the equations, the inability to compare between species or assumptions of the models depreciate their application (Ricklefs 1987). Ricklefs (1987) and Ricklefs and Starck (1998) summarised a method to compare embryonic growth among species using a parabolic growth model whereby;

$$mass = \alpha \, (age)^{\beta} \qquad (1)$$

This equation describes the change in embryo mass using a small number of constants which can be interpreted biologically, without violating the growth curve data (Ricklefs 1987). The parameters α and β can be derived by log transforming the regression of mass and time, providing an equation of the form;

$$\ln (mass) = \ln\alpha + \beta \times \ln(age) \qquad (2)$$

In *S. apama* $\alpha = -16.55$ and $\beta = 2.63$ (Fig. 2.21). This form of the equation can be recast as the allometric relationship between absolute growth rate (dM/dt)) and embryo mass (M): $dM/dt = aM^{b}$, where the parameters a and b describe the growth performance. Integration of the equation provides the increase in mass of the embryo over time; $M = [a(1-b)t]^{1/(1-b)}$ which is of the same form as equation 1; where $\alpha = [a(1-b)]$, and $\beta = 1/(1-b)$. The parameters $\ln a$ and b, which describe the increase in mass of the embryo over time, can then be solved. The growth rate of the embryo at any one time is indicated by $\ln a$, whereas b describes the rate of change of the growth rate with increased size (Ricklefs and Starck 1998). The analysis of *S. apama* embryonic growth produced values of $\ln a = -1.77$, and $b = 0.62$. Unfortunately data on embryonic growth of cephalopods is sparse and an analysis of growth has not been performed on this group of animals, therefore the growth pattern of birds and reptiles was used for comparison. Average values for $\ln a$ and b are respectively -0.95 and 0.724 for birds, and -2.56 and 0.513 for reptiles (Ricklefs and Starck 1998). More negative values of

ln*a* were calculated for slow developing altricial birds, pelagic seabirds and reptiles indicating long developmental times. The ln*a* value for *S. apama* approaches that of reptiles. Reptiles typically display the sigmoidal pattern of growth and precocial development, however precocial birds had high ln*a* values indicating that the growth parameters describing the increase in embryo mass bear no relationship to the developmental state of the neonate (Ricklefs 1987, Ricklefs and Starck 1998). Similarly, in *S. apama*, low ln*a* values were calculated indicating long development, but the growth pattern is exponential. Large *b* values indicate the rate of change in the growth rate is maintained at a high level. However, values of *b* for *S. apama* were relatively small, indicating that the rate of decline in the relative growth rate is greater than in other species (Ricklefs 1987).
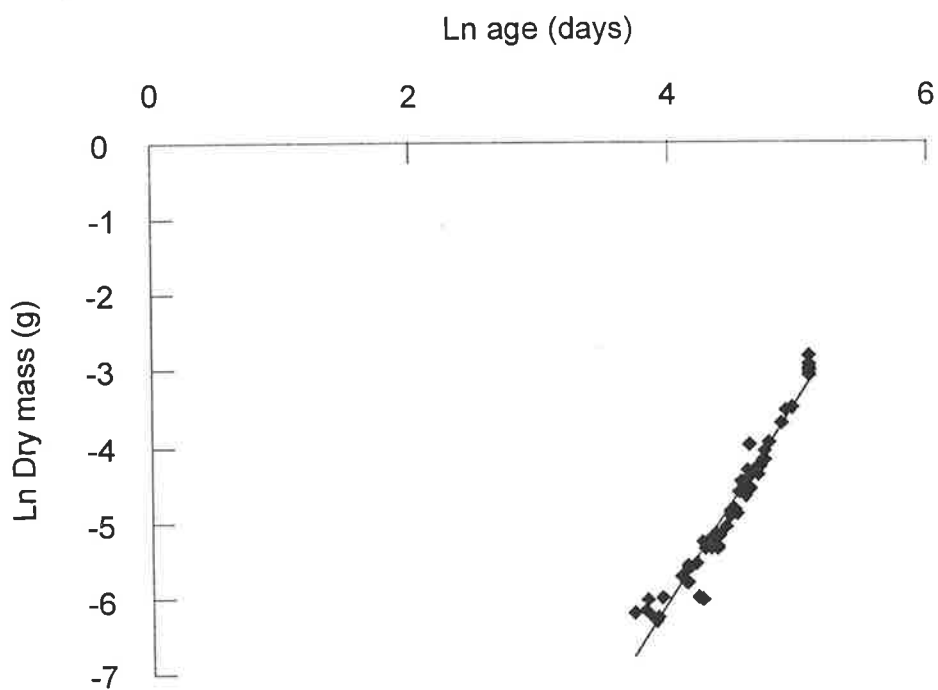
Figure 2.21: Relationship between natural logarithms of embryo mass with age. The linear equation describing the data is: ln(dry mass) = - 16.553 + 2.631 × ln(age), r^2 = 0.95. Data from Figure. 2.14.

The decrease in relative growth rate and increase in mass of the embryo describes development. Relative growth rate declines throughout development as the tissues differentiate and acquire functional capacity (Schmalhausen 1930 in Ricklefs 1987). As tissues mature and become functional, the proportion of water decreases and the density increases, resulting in increased dry mass throughout development (Ricklefs and Starck 1998). Therefore, the increase in dry mass of the embryo with respect to the increase in wet embryo mass provides a measure of developmental maturity. The rate of increase in tissue dry mass (D) can be expressed as a function of tissue wet mass (M), whereby $D = iM^s$ (Fig. 2.22). Tissue dry mass increases throughout development, hence s, the rate of maturation of the developing embryo, is always >1. Rates of tissue maturation (s) correlate inversely with relative growth rate (b) in birds (Ricklefs 1987) and reptiles (Ricklefs and Starck 1998), with low relative growth rates compensated for by high rates of tissue maturation. For *S. apama* the parameters were low ($\ln i = -1.82$ and $s = 1.01$) in comparison to altricial birds ($\ln i = -2.41$, $s = 1.29$) and reptiles ($\ln i = -2.51$, $s = 1.46$) (Ricklefs and Starck 1998) indicating the rate of maturation of tissues is extremely slow. Therefore, b would be expected to be high in *S. apama*, however the value for b was also low (0.62) indicating the relative growth rate is also slow. In pelagic seabirds both b and s are also low, suggesting other mechanisms act on development. Ricklefs and Starck (1998) also found that larger eggs (and therefore large embryos) have a low rate of tissue maturation (s) and consequently low $\ln i$. A long incubation period may result from slow growth of a single tissue or organ (eg. nervous system), limiting the growth rate of other tissues (Ricklefs 1979). 'Optimal' growth conditions may also be better met with slow development, as is seen in the production of larger embryos of birds at lower temperatures (Romanoff 1960).
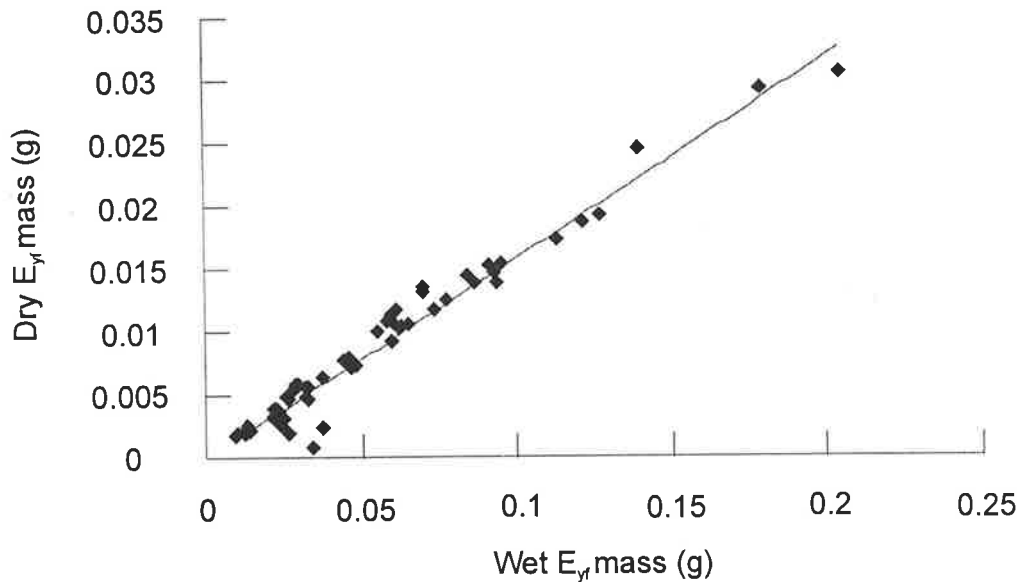
Figure 2.22: The relationship between the internal yolk free embryo (E_{yf}) dry and wet mass. The equation describing the data is; dry mass $= 0.1625 \times$ (wet mass)$^{1.0134}$.

In *S. officinalis,* hatchling size decreases with increased incubation temperature, indicating that reduced incubation period results in smaller hatchlings (Bouchaud 1991). The consumption of yolk and the size of the hatchling is maximised at a temperature of 15°C. The longer developmental period associated with a lower temperature provides optimal growth conditions, resulting in a larger hatchling. Juvenile survival is expected to correlate to hatchling size, as a larger individual should be able to avoid predators, obtain food, and survive through food shortages better than a small individual (Spight 1976, Weatherly and Gill 1987, Hutchings 1991). Therefore 'optimal' incubation temperatures which maximise hatchling size would be selected for. Conversely, hatchling "viability" in *Octopus mimus* was not affected by temperatures of 16-24°C suggesting this species has a wide temperature tolerance (Warnke 1999). Similarly, the incubation temperature of *S. apama* ranges from 12°C in June - July to 18-20°C in October-November, with eggs deposited as late as September. This suggests *S. apama* eggs also have a high temperature tolerance, however, the effects of developmental temperature on the growth trajectory, hatchling size and juvenile viability would need to be assessed to determine if optimal developmental temperatures exist for *S. apama*.

In summary, *S. apama* eggs exhibit a large initial size variation, which is maintained throughout development, resulting in larger hatchlings from larger initial egg size. Development at 12°C requires an incubation period of 160 days. In this period, the egg volume increases by over 180%, and the embryo develops from the large yolk into a miniature adult with an internal yolk reserve that accounts for approximately half of its dry mass. The growth pattern of the embryo reflects the avian exponential growth model, however the rate of growth is much slower than in birds, indicating that metabolism is inherently low. Accompanied with low incubation temperature, this prolongs the developmental period, which necessitates a greater degree of protection in the form of capsule material.

Staging embryonic development

Introduction

A descriptive narrative of the changes in developmental processes of an embryo can be summarised in staging tables. Stages are characterised by the appearance or change in morphology of developmental features. However, since developmental features are often complex and generally change smoothly over time, the applicability of defining 'stages' using objective markers has been questioned (deBeer 1951, Boletzky 1987a). Nevertheless, the development of staging tables is fundamental in experimental embryology (Arnold 1965), can assist researchers in estimating the age or relative maturity of an embryo against a known set of standards, and may be useful in determining environmental impacts on egg development (Blackburn et al. 1998).

One of the most striking differences between cephalopods and most other mollusc classes is the developmental mode. The cephalopod egg is telolecithal, containing a large yolk with a germinal disk at one end. Cleavage is meroblastic and the young hatch as juveniles that resemble miniature adults. Conversely, in most other mollusc eggs cleavage is spiral and the young hatch as larvae. Embryonic development of the cephalopods has been well documented (see references in Lemaire 1970). The most widely used staging tables are those of Naef (Naef 1923, 1928). This included an account of the Northern Hemisphere cuttlefish, *Sepia officinalis*, based on the chronological age of the embryo. Naef's (1928) tables omit cleavage events and consist of 20 stages, which are broadly applicable to many octopod and decapod species (Fioroni 1990). However, since development is temperature-dependent, with decreased developmental duration at greater temperatures, staging by chronological age may miss important developmental events and overemphasise the significance of the development rate (Arnold 1965). Temperature also affects the proportion of time spent at

each stage, with decreased temperature resulting in a shortening of the post-organogenetic phase relative to the preceding phases (Boletzky 1987a). The utilisation of the yolk is also affected by temperature, with more yolk consumed at low temperatures resulting in larger hatching size in *S. officinalis* (Bouchaud and Galois 1990, Bouchaud 1991). The event of hatching has been used as a staging indicator, however, hatching can occur either prematurely, when the embryo discards any remaining yolk as a result of disturbance, or late, if the embryo remains in the capsule when all outer yolk has been absorbed. Thus the timing of hatching is not a reliable indicator of the developmental stage (Boletzky 1987a). Therefore, staging tables should be based on morphological events rather than chronological age and must clearly state the conditions of development to enable comparisons between investigators. Staging tables based on morphological events have been complied for the squid *Loligo pealli*, (Arnold 1965), for *S. officinalis* (Lemaire 1970), and more recently for a number of other species (Segawa *et al.* 1988, Baeg *et al.* 1992, Vecchione and Lipinski 1995, Blackburn *et al.* 1998), but not for the giant Australian cuttlefish *Sepia apama*.

The embryonic development of *S. apama* is expected to be similar to *S. officinalis*. Lemaire (1970) divided the embryonic development of *S. officinalis* into three periods consisting of cleavage (stage 1-9), gastrulation (stage 10-17) and organogenesis (stages 18-30). The first two periods concern meroblastic cleavage and the formation of the yolk syncytium. The embryo proper becomes identifiable at stage 14 when thickenings (placodes) appear, distinguishing the future regions of the head, eyes, mantle and arms. By stage 18 (the beginning of organogenesis) the embryo is raised from the yolk and has basic, but easily distinguishable features. More than half of the development time is spent in stages xv - xx (Naef) or 27-30 (Arnold), with over 80% of the growth occurring from stages xviii-xx (Naef) or 28-30 (Arnold) (Boletzky 1983a). Since a far greater proportion of the developmental duration involves the latter developmental stages, these deserve greater attention. In this study I have described the developmental stages of *Sepia apama* during organogenesis

(stages 18-30) including descriptions of embryonic activity and behaviour, as an initial step to develop a staging table specific to *S. apama*.

Methods

Eggs were incubated in darkness in flow through aquaria maintained at 12°C. Embryos were removed from the egg following measurements of morphology and oxygen consumption (Chapter 2 and 4), and dorsal mantle lengths were measured with a micrometer and dissecting microscope. Representative samples of embryos were examined using light microscopy and an image was drawn from the living material with the aid of a camera lucida. In addition, photographs were taken to supplement the drawings using a camera mounted on a microscope.

Results

A generalised account from the literature of gonad development, fertilisation and embryonic development of the decapods is provided for completeness, supplemented with a description and staging table of the latter period of development specific to *S. apama*.

Gonad development and fertilisation

Within the ovary of *S. apama* different stages of egg development are present (Fig. 3.1) indicating that spawning occurs more than once during a breeding season. Mature eggs consist of a large yolk surrounded by a vitelline membrane inside of a chorion membrane. The eggs are released into the oviduct where they are coated with a series of spirally coiled jelly layers secreted by the oviducal glands, nidamental glands and accessory nidamental glands (Boletzky 1987a). Copulation occurs when the male withdraws spermatophores from his genital opening and transfers these using a specialised tentacle, the hectocotylus, to the seminal receptacle, a small pouch on the ventral buccal membrane, which secretes a sperm

suppressant (Arnold and Williams-Arnold 1977). The sperm must penetrate the many jelly layers surrounding the egg to reach the micropyle and gain entry to fertilise the egg.
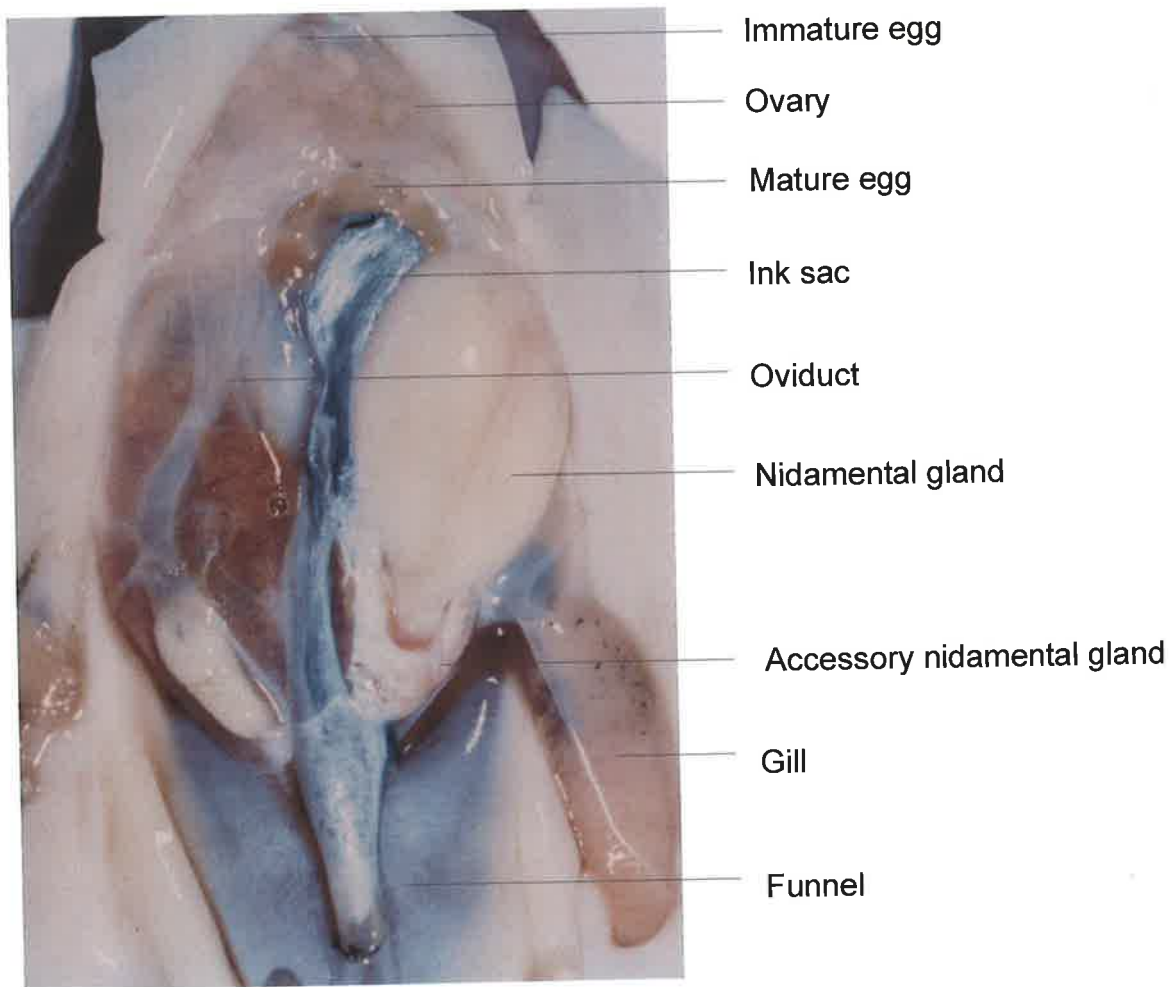
Figure 3.1: Dissected ventral mantle of female *Sepia apama*. The nidamental gland has been removed from the left-hand side to expose the oviduct. The ovary contains eggs at different developmental stages, which indicates that egg laying is iteroparous. Also note the pale orange colour of the accessory nidamental gland which is probably an indicator of symbiotic bacteria which are introduced into the capsule material during egg formation.

Cleavage

Development proceeds by the formation of a blastodisc beneath the micropyle at the animal pole (Arnold and Williams-Arnold 1977). The blastodisc "recruits" cytoplasm from the

periphery resulting in cytoplasmic streaming. The blastodisc undergoes meroblastic cleavage. During the 3rd cleavage, a plasma membrane forms between the cytoplasm and the yolk. During 4th cleavage two groups of cells are distinguished; a small group and a surrounding syncytium. Continued cleavage separates these two cell groups such that a central layer of cells is formed surrounded by a peripheral syncytium connected via membranes underneath the inner group of cells or blastoderm. A ring of cells forms on top of the blastoderm cells creating a depression in the yolk and the formation of a papilla. This ring grows inwards covering the papilla (Arnold and Williams-Arnold 1977).

Gastrulation

Three cellular layers are distinguishable; the inner syncytial yolk epithelium (responsible for digestion of the yolk early in development), and a middle and outer cellular layer (the future embryo). This multi-layered blastoderm spreads to cover the cap of the yolk delineating the future organogenic region of the embryo, whilst the remaining yolk becomes cellulated with the middle layer of cells and inner syncytial layer, which gives rise to the outer yolk sac (Arnold 1971).

Organogenesis

Organogenesis begins with the formation of placodes or raised thickenings of epithelial cells, delineating the future regions of the mouth, cephalic lobe, eye, mantle, shell, gills, funnel and arms (Lemaire 1970, Fioroni 1990). Between the yolk epithelium and the outer layer of cells of the external yolk sac a large haemal space develops. Within this blood sinus muscle bands develop which traverse the haemal space and connect the yolk epithelium to the outer layer of cells of the yolk sac. The contraction of these muscle cells causes contraction of the external yolk sac which circulates the blood in the haemal space and through the developing vessels. Yolk platelets are broken down and the nutrients transferred to the haemal space (Arnold 1971). Yolk is transferred during development to two internal yolk sacs located

anteriorly and posteriorly beneath the mantle. The internal yolk sacs have associated blood sinuses in the head region (the cephalic sinus) and internal to the funnel folds and gills (posterior sinus), and are broadly connected to the haemal space of the external yolk sinus. As development proceeds the anterior blood sinus gradually disappears, and the posterior sinus becomes the abdominal vein. The circulatory function of these organs is superseded by the development of branchial hearts at the base of each gill, systemic hearts, and the aorta and branchial veins, which unite to form the adult blood circulatory system, whilst the digestive function is replaced by the formation of the liver (Arnold 1971). Cilia develop on the yolk and embryo during organogenesis. Yolk cilia are respiratory in function, however, the skin replaces the respiratory role of the yolk cilia, whilst the mantle cilia circulate the PVF (Ranzi 1926). In *Sepia* ciliary tufts appear only on the posterior dorsal mantle, whilst they occur on both the dorsal and ventral mantle surfaces of squid (Boletzky 1987a). During the latter stages of development a specialised hatching device, the organ of Hoyle, develops on the dorsal posterior mantle. The organ of Hoyle is anchor shaped, with a large vacuole in which hatching enzymes are stored. During hatching, the organ is positioned against the chorion and the enzymes are released. The enzymes digest the chorion membrane, whilst the internal pressure of the perivitelline fluid and muscular contractions of the mantle release the hatchling from the confines of the capsule (Boletzky 1987a).

Sepia apama *organogenesis*

The following describes stages of organogenesis of *Sepia apama* embryos incubated at 12°C, based upon the staging tables of Lemaire (1970).

Stage 18: The mouth, cephalic lobe, eye, mantle, shell, gills, funnel and arms become identifiable as placodes or raised thickenings of epithelial cells. These features become more marked and raise from the yolk surface, whilst the shell gland reduces in size. The arms show paired primordia (Fig. 3.2).
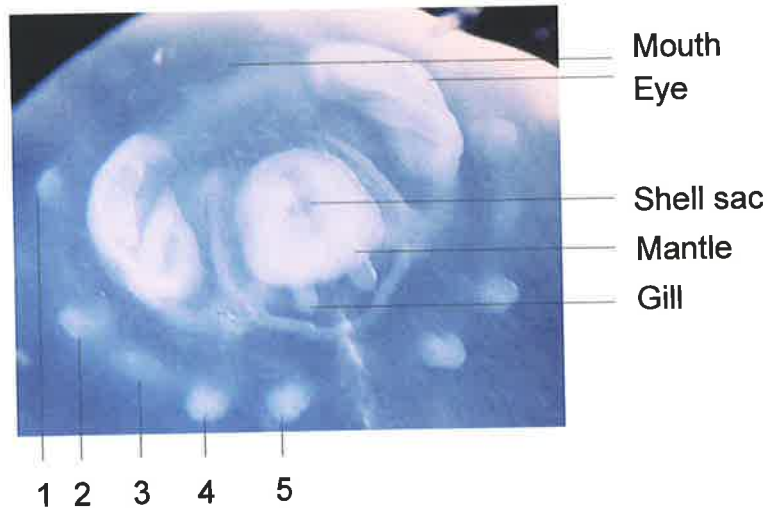
Figure 3.2: Stage 18 embryo showing raised placodes identifying future regions of organ formation. Numbers 1-5 indicate arm rudiments.

Stage 19: The embryo becomes distinct from the yolk as the organs enlarge and become more defined. The shell gland on the disc-shaped mantle closes and the ventral edges begin to turn downwards. The gills are pedicelate.

Stage 20: The shell gland closes completely and fins on the mantle become evident. The cup-shaped mantle covers the base of the gills. Protuberances at the back of the eye indicate the optic lobe primordia. Arms are well developed and no longer appear paired.

Stage 21: The mantle covers $^1/_3$ of the gills. The gills begin to develop laminae. The funnel folds curve inwards at the anterior end. Suckers begin to be evident on arm 4 (future prehensile tentacle) in single row. Lens primordia appear. Age = 40 days, DML = 1.1 ± 0.03 (8) (Fig. 3.3 and 3.4).

Stage 22: The mantle extends to cover ½ of the gills. Gills have eight laminae as raised ridges transverse to the gill. The funnel folds curve inwards but are not fused. Suckers in single row on arm 4, and just becoming visible on arm 5. Lens rod shaped. Age = 41 days, DML = 1.2 ± 0.03 (3).

Figure 3.3: Photographs (top row - dorsal, bottom row - ventral) of selected stages of embryonic development of *Sepia apama*. Bars represent 1mm for stage 21 - 25, and 2 mm for stage 26 and 28.
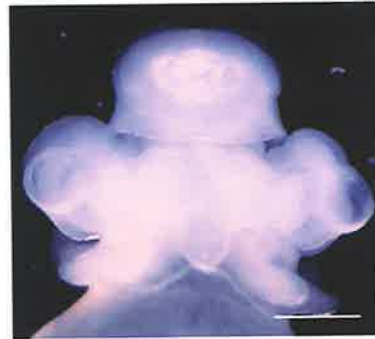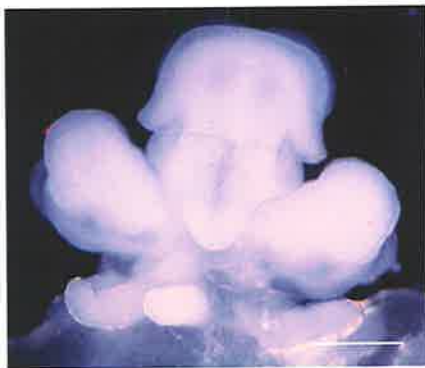
Stage 26

Stage 21

Stage 23
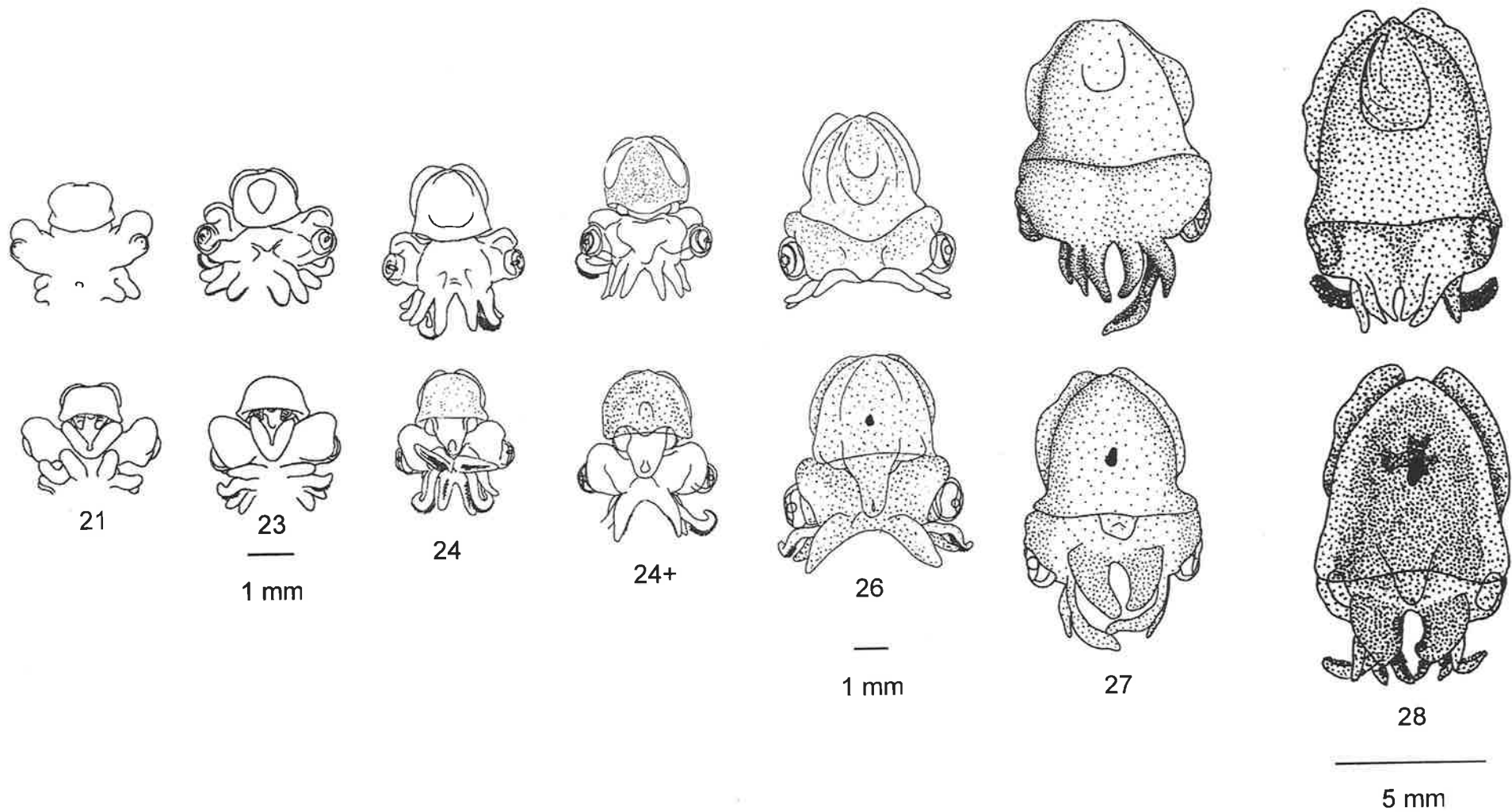
Stage 25

Stage 28

Figure 3.4: Drawings (top row - dorsal, bottom row - ventral) of developmental stages of *Sepia apama*.
The scale bar for stages 21, 23 and 24 is below stage 23, and for stages 24+, 26 and 27 the scale bar is below stage 26.

Stage 23: The mantle covers $^2/_3$ of the gills. Anterior $^1/_3$ of funnel is fused, forming a triangular opening between the funnel and mantle. The Organ of Hoyle appears. Suckers beginning on arms 1, 2 and 3. No pigmentation. (Fig. 3.3 and 3.4). Age = 44 days, DML = 1.4 ± 0.02 (15).

Stage 24: The mantle completely covers the gills. The gills have 11-12 laminae as raised plates. The funnel is fused and the triangular opening between the funnel and mantle is smaller. Suckers occur on all arms. Lens becoming less elongate and more rounded. Pale orange colouration in retina and chromatophores on ventral mantle surface. Ciliation of yolk enables embryo to move in petri dish. Mantle contracting. Age = 53 days, DML = 1.8 ± 0.04 (20) (Fig. 3.4).

Stage 25: Pale orange chromatophores on dorsal mantle and bright orange coloration on retina. Lens almost spherical. Age = 63 days, DML = 2.3 ± 0.04 (31) (Fig. 3.3).

Stage 26: Chromatophores on dorsal surface and ventral mantle. Lens spherical. Ventral surface of eye partially covered by secondary cornea. Ink sac evident on ventral mantle. Head region wider than mantle diameter. Age = 77 days, DML = 3.2 ± 0.06 (29) (Fig. 3.3 and 3.4).

Stage 27: Eye half covered by secondary cornea. Retina colouration deeper orange-red. Chromatophores on dorsal mantle orange-brown in colour, larger and more numerous. Diameter of head region approximating mantle diameter. Air filled chamber(s) in cuttlebone. Age = 90 days, DML = 4.3 ± 0.05 (45) (Fig. 3.4).

Stage 28: Eye completely covered by secondary cornea. Retina is orange/bronze. Three air-filled cuttlebone chambers. Chromatophores on arms. Orange smearing around ink sac. Ability to release ink. Age = 105 days, DML = 5.8 ± 0.11 (38) (Fig. 3.3 and 3.4).

Stage 29: Cuttlebone with six chambers. Ejects ink if agitated. Retina darker copper/brown. Chromatophores on mantle, head and arms darker red-brown. Iridocytes in bilaterally symmetrical pattern on mantle, head and arm 1. Age = 127 days, DML = 8.6 ± 0.11 (54).

Stage 30: W-shaped black pupil. More numerous iridocytes on mantle, head and arms. Ten cuttlebone chambers. Epidermal lines develop on arms and head. Age = 145 days, DML = 10.7 ± 0.12 (19) (Fig. 3.5).

In the latter two stages the head and body are completely pigmented. Chromatophores are not utilised whilst within the capsule, but are functional as observed during premature removal from the capsule. Iridocytes begin to appear at first on the dorsal mantle at the anterior periphery of the cuttlebone, the sides of the ventral mantle and two between the eyes as well as one each on arm 1. Progressively more iridocytes appear on the mantle, head and arms (Fig. 3.5). In *S. officinalis* epidermal lines of cells occur on each side of the head extending to the arms. These ciliated sensory epidermal cells are surrounded by non-ciliated accessory cells and provide a function similar to the lateral line of fish (Sundermann 1983, Budelmann and Bleckmann 1988). The lines observed on *S. apama*, between the eyes and extending along the arms (Fig. 3.5) are likely to perform a similar function.

Behaviour and hatching

With the exception of pumping water past the gills from very early in development and the occasional flutter of the fins somewhat later, the embryo essentially remains quiescent throughout the majority of development. Only from stage 29 onwards does any increase in activity occur. Prior to hatching the embryo rapidly pumps water through the funnel, lifts itself and attached yolk from the bottom of the capsule and moves around the circumference of the capsule with the dorsal surface oriented outwards. Activity occurs in short bursts lasting 1-2 s, accompanied by increases in mantle contraction frequency and amplitude

(Chapter --.   also initiated when the capsule is mechanically disturbed and may

occasionally result ...   ? embryo ejecting ink into the egg. If disturbed sufficiently the

embryo may hatch with a considerable amount of external yolk remaining (Chapter 5).

Increased activity culminates in hatching, whereby the embryo detaches any remaining yolk

and positions the organ of Hoyle against the upper half of the capsule. In a matter of seconds

the organ of Hoyle releases enzymes which dissolve the chorion and capsule, creating a slit

through which the embryo exits, mantle first (Fig. 3.6).
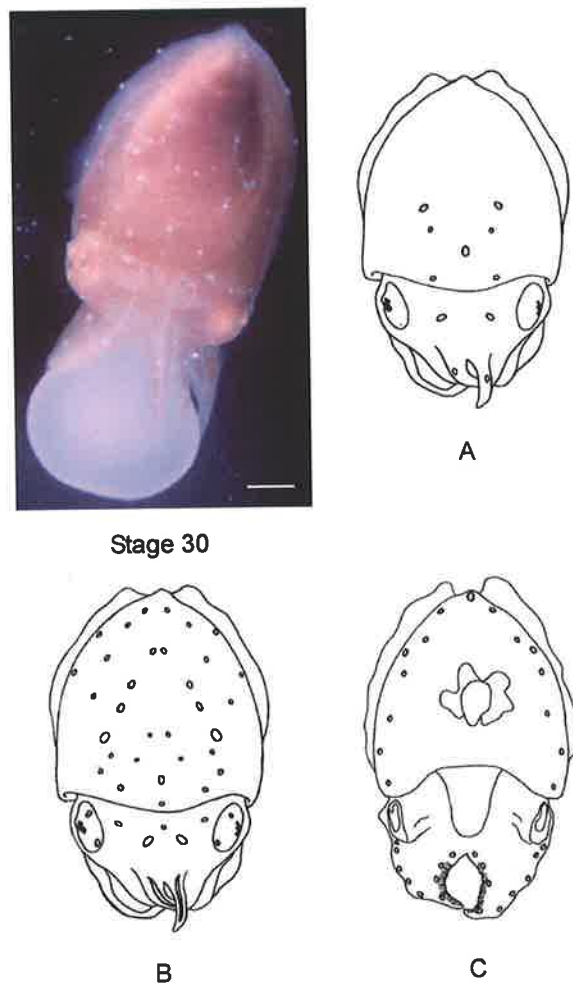
Stage 30

Figure 3.5: Stage 30 embryo with attached yolk (scale bar = 2 mm) and diagrams showing the development of the iridocyte pattern. Initially, iridocytes develop on the dorsal mantle and head (A), these increase in number in a symmetrical pattern (B), and also become evident on the ventral mantle (C).
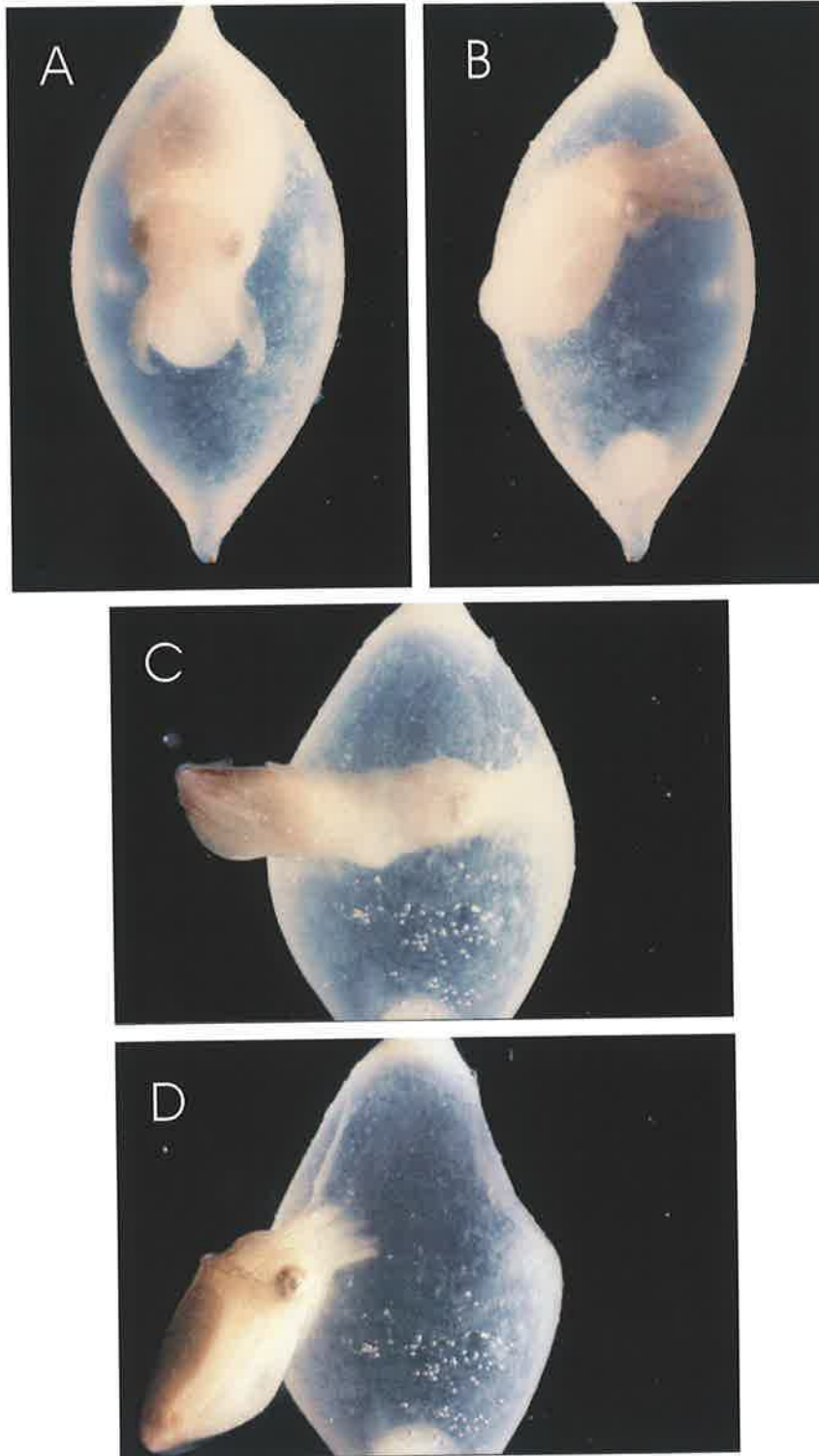
Figure 3.6: Hatching sequence of *Sepia apama*. The embryo with attached yolk (A), disengages the yolk and positions the organ of Hoyle against the capsule wall (B), prior to exiting the capsule (C and D).

Discussion

If the differences in yolk volume are taken into account, the pattern of development throughout the cephalopods is uniform (Arnold and Williams-Arnold 1977). As in *S. officinalis* a large proportion of the developmental period of *S. apama* is occupied by organogenesis. At 12°C, organogenesis accounts for 120 days or 75% of the developmental period, approximately half of which is spent in the latter three stages of development (Fig. 3.7). The developmental stages 18-24 proceed most rapidly, however dorsal mantle length
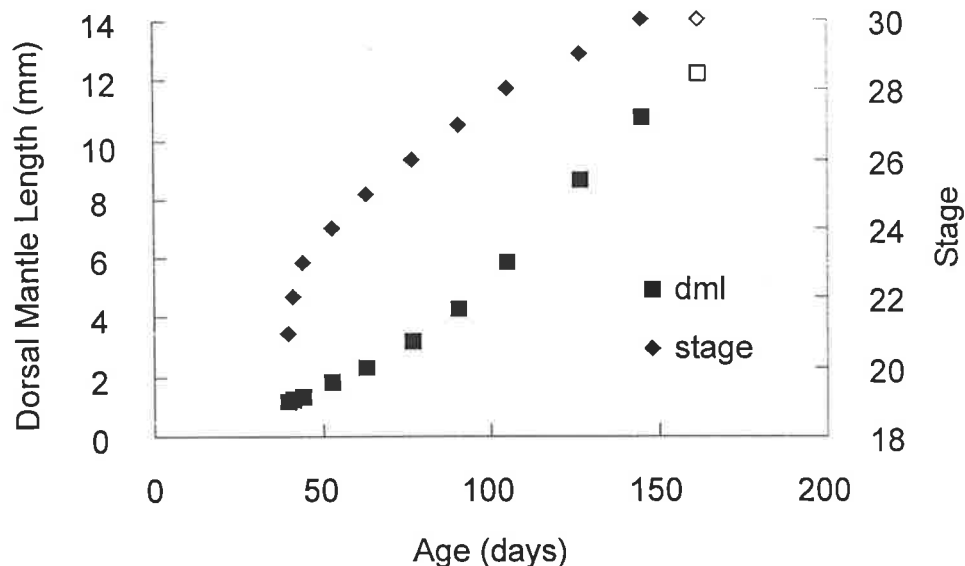
Figure 3.7: Changes in dorsal mantle length (DML) with development at 12°C, and associated developmental stages. Open points represent hatchlings, n= 8, 3, 15, 20, 31, 29, 45, 38, 54, 19, 15 respectively.

increases only slowly (Fig. 3.8). Thereafter, the dorsal mantle length accelerates, but the embryo has acquired the majority of the features of a hatchling, and subsequent changes in size and pigmentation are gradual. This developmental pattern is unlikely to be indicative of the field situation, since the eggs are exposed to increasing temperatures nearing the latter stages of development in the field. In *S. officinalis* as incubation temperature decreases the proportion of time spent in the post-organogenic stages decreases relative to the preceding
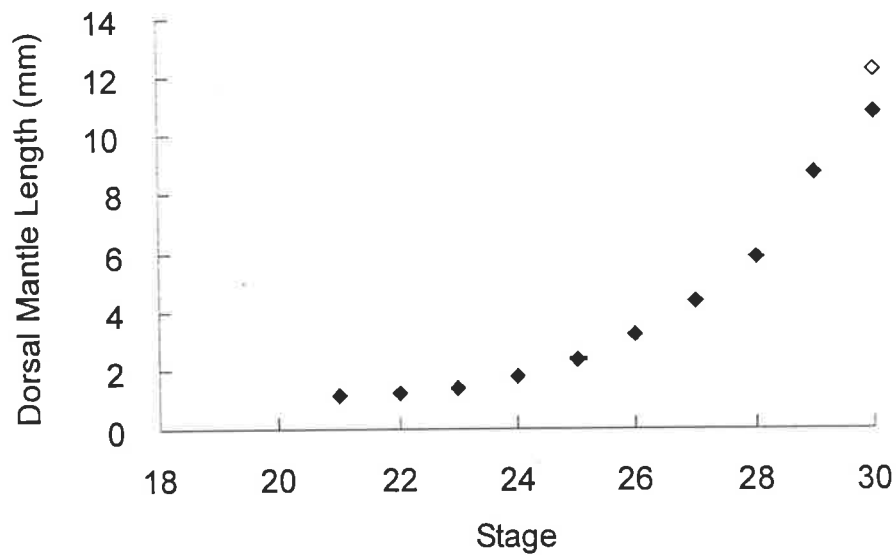
Figure 3.8: Relationship between dorsal mantle length and developmental stage of *Sepia apama* embryos at 12°C. Open point represents hatchlings.

stages and yolk consumption increases (Boletzky 1987a), resulting in a larger hatchling (Bouchaud and Galois 1990). *S. apama* eggs exposed to increasing temperatures in the field may therefore spend a relatively greater amount of time in the latter developmental stages in comparison to eggs maintained at constant temperature. Thus the 75% of time spent in organogenesis is likely be an underestimate in comparison to eggs developing in the field. Conversely, the higher field temperatures would reduce the absolute developmental period. The 160 day development period of *S. apama* at 12°C is similar to the developmental period of *S. officinalis*, which increases as temperatures decrease from 40-45 days at 20°C to 80-90 days at 15°C (Boletzky 1983a). Provided that the effects of temperature on developmental duration and succession are taken into account, the staging table developed for *S. apama* can be useful for both intraspecific and interspecific comparisons.

Gas exchange in eggs

Introduction

The dissolved oxygen content of water is a function of the oxygen solubility and oxygen partial pressure (PO_2). Oxygen solubility varies only slightly, decreasing with both increased temperature and salinity. The partial pressure of oxygen in air equilibrated water is equivalent to the partial pressure of oxygen in saturated air (at 1 atmosphere = 20.7 kPa). However, the capacity of water to hold oxygen, that is its solubility or capacitance (βO_2) is 1/30 that of air (0.5 - 1 % compared to 21% (Graham 1990)), and the diffusivity of oxygen in water (DO_2) is 7500 times slower in comparison to air (2×10^{-5} compared to 0.198 cm^2 s^{-1} (Dejours 1988)). This results in a Krogh's diffusion coefficient (KO_2) in water 1/225 000 that of air. These physical properties of water result in thicker diffusion boundary layers, which limit the accessibility to oxygen for respiration (Graham 1990). In addition, aquatic environments are subject to greater variation in oxygen content, owing to diurnal swings in oxygen production and consumption (Graham 1990, Seymour 1999), however these are generally confined to closed water bodies without adequate mixing, as opposed to open oceans which approximate air saturation (Graham 1990). The consequences for organisms respiring in aquatic habitats are slow rates of gas exchange and possible exposure to large fluctuations in oxygen content. These attributes of water may impose limitations on respiratory systems and hence influence life histories (Strathmann 1990).

Eggs developing in an aquatic habitat are commonly enclosed within a gelatinous matrix which essentially represents an unstirred water barrier through which oxygen must diffuse to reach the embryo. The low diffusion coefficient of oxygen in water means that diffusion of oxygen through egg jelly is slow. Consequently, aquatic eggs are generally expected to be smaller, require less oxygen, have shorter developmental periods and therefore hatch at an

earlier stage in comparison to eggs developing in air (Salthe and Duellman 1973, Bradford 1990).

The principles of gas exchange have been established in many types of eggs, and can be applied and adapted for the eggs of *Sepia apama*. The diffusion of oxygen is driven by a partial pressure gradient between the external (PO_{2out}) and internal (PO_{2in}) environments of the egg, established as a result of the oxygen consumption rate of the embryo ($\dot{V}O_2$) and the conductance of the capsule (GO_2) (Seymour and Bradford 1987). Thus oxygen uptake by the embryo can be described by the Fick equation whereby:

$$\dot{V}O_2 = GO_2 \times (PO_{2out} - PO_{2in}) \tag{1}$$

GO_2 ($\mu l\ h^{-1}\ kPa^{-1}$) depends on the effective surface area of the capsule (ESA - determined as the geometric mean of the inner and outer radii of the capsule), its thickness (X), and Krogh's coefficient of diffusion (KO_2 ($cm^2\ min^{-1}\ kPa^{-1}$)), itself the product of diffusivity (DO_2) and capacitance (βO_2) (Seymour 1994):

$$GO_2 = KO_2 \times ESA\ /\ X \tag{2}$$

$$KO_2 = DO_2 \times \beta O_2 \tag{3}$$

Provided that PO_{2in} (kPa) remains high, the $\dot{V}O_2$ ($\mu l\ h^{-1}$) remains dependent only upon the metabolic requirements of the embryo. However, if either GO_2 is too low or PO_{2out} decreases, then PO_{2in} decreases to a point where $\dot{V}O_2$ becomes diffusion limited (Seymour and Bradford 1995).

GO_2 is directly related to KO_2 and ESA, and inversely related to the thickness of the capsule. At a given temperature the KO_2 of the capsule is a constant, however this value varies with the characteristics of the jelly, and in frog egg jelly is estimated to be 76% of pure water at 20°C (Seymour and Bradford 1987). In gelatinous coated eggs, the GO_2 increases

throughout development as a result of water uptake into the perivitelline space which increases the surface area for gas exchange and reduces the capsule thickness and diffusive distance for oxygen. Increased GO_2 is necessary to facilitate the increased $\dot{V}O_2$ of the developing embryo, however the increases in GO_2 may not fully compensate for the increased $\dot{V}O_2$, resulting in $\dot{V}O_2$ becoming diffusion limited. Changes in the GO_2 have been shown to counteract the increased $\dot{V}O_2$ throughout development in a terrestrial breeding frog, *Pseudophryne bibronii*, where PO_{2out} is consistently high (Seymour and Bradford 1987). However, an aquatic breeding frog with similar sized eggs, *Crinia georgiana*, was shown to experience oxygen limitation in late stage embryos as a result of the high metabolic demands and low ambient PO_2 (Seymour and Roberts 1995).

The consumption of oxygen within closed water bodies results in the decline of the water PO_2, the effects of which can be exacerbated with increased temperatures which further decrease the PO_2 of the water. Adequate mixing is necessary to reduce local PO_2 depressions or boundary layers, around the egg. These boundary layers are created when water velocities decrease, resulting in a build-up of oxygen depleted, carbon dioxide rich water around the egg. Boundary layers effectively increase the diffusive distance for oxygen, slowing the rate of exchange of materials between the organism and its environment (Pinder and Feder 1990). Therefore it is important to select depositional environments with a high PO_{2out} to reduce the likelihood of diffusive limitation of $\dot{V}O_2$.

The embryo may respond to decreased PO_{2in} by increasing the gill ventilation rate or magnitude (and hence $\dot{V}O_2$) in an attempt to counteract the declining PO_{2in}. Embryos which maintain their $\dot{V}O_2$ independent of PO_2 have classically been referred to as oxygen regulators, whilst those whose $\dot{V}O_2$ relates directly to changes in PO_2 are oxygen conformers (Herreid 1980). Rarely, however are animals either of these two extremes and often their response to environmental PO_2 will vary depending on the environmental conditions and the physiological state of the individual (Herreid 1980). Therefore the response of the embryo to PO_2 will change as the embryo develops and its requirements change.

Conditions favouring high PO_{2in} are eggs with low metabolic rates, thin capsule walls and large surface areas, deposited in regions with adequately mixed water. Small egg size achieves the physiological and morphological objectives, as ovum volume correlates with $\dot{V}O_2$, and the capsule thickness need only be thin to support a small egg, whilst, relative to the volume, the ESA remains large (Seymour and Bradford 1995). On the contrary, a large egg generally produces a larger embryo, with an associated higher metabolic rate and longer developmental duration necessitating a thicker capsule for protection. The maximum size attainable prior to diffusion limited $\dot{V}O_2$ is estimated to be less than 1 ml volume in amphibian eggs (Seymour and Bradford 1995). Aquatic developing mollusc eggs are rarely larger than 10 mm in any one dimension and are usually less than 1 mm, have short developmental duration and often hatch at pre-metamorphosed stages (Strathmann 1987, Smith *et al.* 1989). Within the cephalopoda egg size may be larger, with a longer developmental duration resulting in a fully developed hatchling (Table 4.6). The eggs of *S. apama* are very large and are expected to represent the boundary of diffusion limitation for gas exchange under natural developmental conditions.

The intention of this chapter is to examine the oxygen consumption of S*epia apama* eggs throughout development and relate these to the changes in egg morphology to determine whether the embryonic $\dot{V}O_2$ is diffusion limited. Diffusion limitation can be identified by the response of the $\dot{V}O_2$ to changes in ambient PO_2 and by comparisons of late stage embryonic and hatchling $\dot{V}O_2$. Changes in the convection rate may also identify stressful conditions. The changes in $\dot{V}O_2$ throughout development provide insight into the mode of development and enable quantification of the total oxygen consumed in the production of a *S. apama* hatchling. The implications of gas exchange for egg and egg mass design are discussed.

Methods

Oxygen consumption of the eggs

Oxygen consumption was measured at 21d intervals in six egg groups of similar size. At each interval, twelve eggs were selected from one egg group and transferred to aquaria in a constant temperature room at 12°C at the University of Adelaide, South Australia. Individual eggs or hatchlings were placed into 25 or 50 ml glass syringes (hereafter 'chambers'), sealed with 3-way stop-cocks and filled to 10-15 ml with autoclaved seawater at PO_2 approximating 15, 21, 25 and 35 kPa, with three replicate eggs per partial pressure treatment. Chambers were placed in a shallow tray with circulated water maintained at 12°C by a Haake FK thermocirculator. The same water was circulated through a Radiometer D616 glass jacket containing a Radiometer E5047 oxygen electrode, connected to a Radiometer PHM 72 Mk 2 Digital Acid-Base Analyser. The electrode was calibrated with a sodium sulphate-borax PO_2 - zero solution (Tucker 1967) and air-equilibrated water at 12°C, with 100% saturation (PO_2) calculated using the ambient atmospheric pressure (P_b) and saturated water vapour pressure (P_{H_2O}), whereby; $PO_2 = 0.2094 \, (P_b - P_{H_2O})$ Torr (mm Hg). The eggs were allowed to equilibrate within the chambers for 30-120 min prior to beginning sampling (longer equilibration period for younger eggs). At intervals of 0.5 - 3 h, the chambers were stirred by attaching a 2 ml syringe to the stopcock without admitting air and withdrawing and reinjecting water three times to mix the water in the chamber. Following stirring, a 0.5 ml sample was withdrawn from the chamber and injected into the oxygen electrode chamber. The electrode was allowed to equilibrate for 45 s prior to recording a reading. Between each sample the electrode was flushed with air equilibrated fresh water at 12°C. Eggs were enclosed within chambers for a duration of 4-12 h (longer interval for younger eggs) and control chambers filled with 10 ml of autoclaved seawater were run simultaneously. On the following day the eggs were dissected to remove the capsule. The

capsules were placed in the respirometry chambers and measured in the same manner as the whole eggs or hatchlings. Embryonic $\dot{V}O_2$ was not measured independently of the capsule as embryos removed from the chorion died, and if the capsule was removed from the chorion and contents, the chorion invariably ruptured. Oxygen consumption rates for the whole egg and for the capsules were calculated from the oxygen capacitance of seawater (Dejours 1981) accounting for the changing water volume of the syringe. Since the embryonic oxygen consumption rate could not be determined directly, the difference between the whole egg and the capsule oxygen consumption was calculated and defined as the embryonic consumption.

Respirometry chambers were periodically stirred rather than rocked as this can stress the organism leading to high metabolic rates (Rombough 1988b). The accuracy of measurements by sampling closed circuit respirometry with periodic stirring was checked independently using a closed circuit respirometry chamber with the electrode inserted into the chamber and stirred continuously with a magnetic stirrer.

The eggs dissected for capsule $\dot{V}O_2$ measurements were used for morphological measurements (Chapter 2) in order to calculate capsule oxygen conductance (GO_2), Krogh's diffusion coefficient (KO_2) and the partial pressure difference across the capsule (ΔPO_2), and to enable comparisons between these parameters and the morphological data.

Perivitelline PO$_2$

The internal PO_2 of the PVF was measured by inserting a Diamond General 768-21R combination needle oxygen electrode supported by a micromanipulator through the capsule into the PVF, whilst the egg was in air equilibrated seawater. The electrode was allowed to equilibrate within the egg for 45 s prior to a reading being recorded on a Radiometer PHM 71 Mk 2 Acid-Base Analyser. The electrode was calibrated with nitrogen-equilibrated and air-equilibrated seawater. The saturation level was set for air saturation at 12°C and ambient atmospheric pressure.

Mantle contraction frequency

Late stage eggs were placed into a deep petri dish containing constantly aerated seawater. The temperature of the seawater was controlled by immersing the dish in a container with circulating water maintained at constant temperature by a Haake FK thermocirculator. Each egg was rested on a ring of plastic tubing to maintain its position near to the wall of the petri dish. The egg was back lit and allowed to acclimatise to the conditions for at least 30 min prior to counting the number of mantle contractions during 1 min. Four replicate counts were recorded in succession. The eggs were subjected to temperatures of 15, 20, 15 and 18°C sequentially, to ensure changes in mantle contraction frequency were not a function of time. In a second experiment the eggs were exposed to ambient, high, ambient and low PO_2 sequentially by mixing oxygen or nitrogen to the air bubbled through the seawater. The PO_2 was returned to ambient conditions to ensure the embryos were responding normally and had not deteriorated over time.

Results

Oxygen consumption

A typical set of results from a respirometry run shows declining PO_2 with time for 12 eggs subjected to varying initial PO_2 (Fig. 4.1). The change in chamber PO_2 was converted into rates of oxygen uptake ($\dot{V}O_2$) by taking into account the changing volume of the syringe, the capacitance of the water, the time interval between readings in addition to consumption by control chambers and electrode drift. The $\dot{V}O_2$ within the control chambers averaged 0.267 ± 0.08 μl h^{-1} for 56 separate chambers on different days. The control consumption rate for each day was subtracted from the $\dot{V}O_2$ of the whole eggs, capsules or hatchlings. Electrode drift was taken into account by determining the difference between expected and measured

PO$_2$ for air equilibrated fresh water at each time interval and adding this to the PO$_2$ of the experimental chambers. The individual rates were averaged over the entire experimental period and plotted against the average PO$_2$ for the entire experimental period (Fig. 4.2).
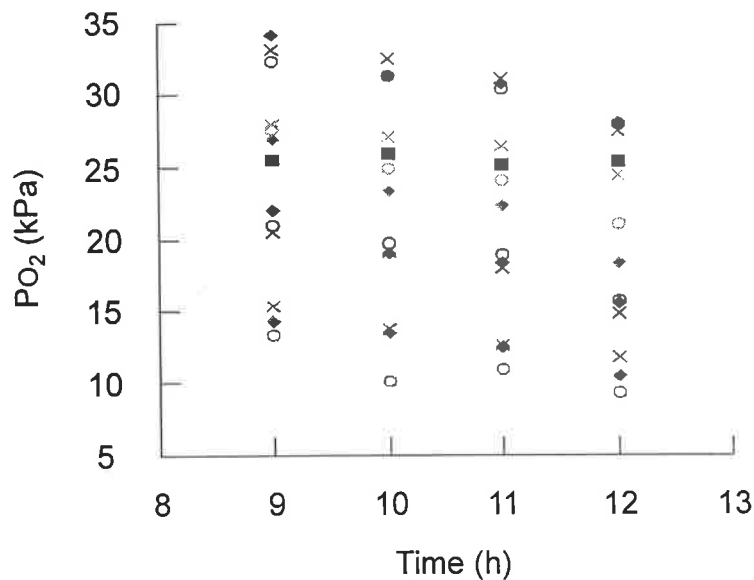
Figure 4.1: Examples of the changes in the PO$_2$ in closed respirometry chambers containing individual eggs. Three replicate eggs (different symbols) per partial pressure treatment (different shades), resulting in 12 eggs plus a control chamber (black squares) were measured at one time.

Comparison of methods

The V̇O$_2$ of late stage eggs within periodically stirred chambers (20.3 ± 1.52 (10) µL h^{-1}) was significantly lower (p<0.001) than the V̇O$_2$ of eggs measured in constantly stirred chambers (30.54 ± 1.88 (10) µL h^{-1}) at 15°C. On average the periodically stirred rate was 67.5% of the constantly stirred rate. The effect of constant stirring was not measured throughout development, but such conditions are not expected to replicate the natural environment (see Discussion), therefore the periodically stirred measurements have been used henceforth, unless stated otherwise.
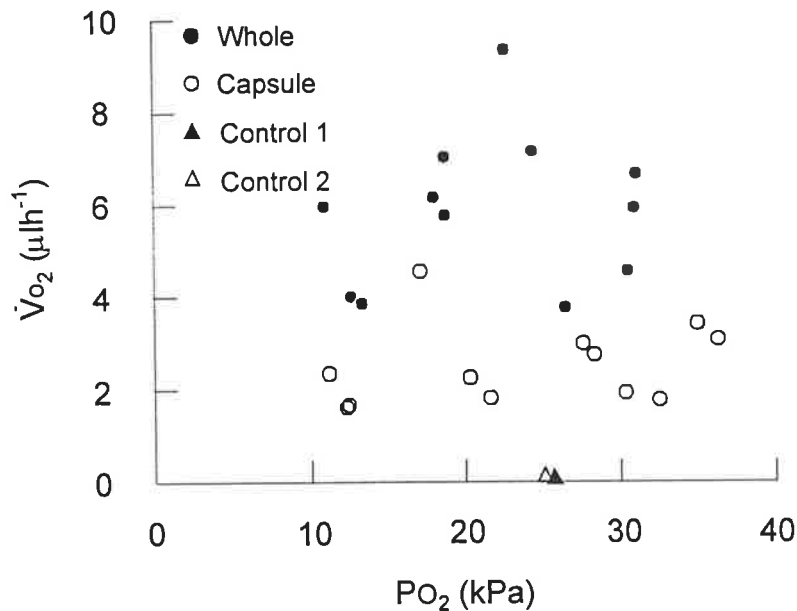
Figure 4.2: Example of the oxygen consumption rates ($\dot{V}O_2$) of whole eggs, capsules and control chambers at varying PO_2.

Table 4.1: Linear regression equations for changes in $\dot{V}O_2$ with PO_2 for whole eggs and capsules at different age categories. NS = not significant.

age category	Average age (days)	Regression equation ($y = PO_2$, $x = \dot{V}O_2$)		r^2	n	p
0-39	11	whole	y = 0.0283x + 1.1454	0.1894	43	<0.01
		capsule	y = 0.0172x + 1.3370	0.0579	43	NS
40-59	48	whole	y = 0.0348x + 1.5950	0.1617	47	<0.01
		capsule	y = -0.0161x + 2.1538	0.0486	47	NS
60-79	70	whole	y = 0.0493x + 2.3329	0.1028	50	<0.05
		capsule	y = 0.0094x + 1.7797	0.0099	49	NS
80-99	90	whole	y = 0.0671x + 2.6950	0.1429	71	<0.01
		capsule	y = 0.0195x + 1.7554	0.0496	70	NS
100-119	110	whole	y = 0.0770x + 4.1360	0.0344	41	NS
		capsule	y = 0.0259x + 1.6052	0.0298	41	NS
120-139	130	whole	y = 0.0788x + 5.0376	0.0374	45	NS
		capsule	y = -0.0080x + 1.9489	0.0058	47	NS
140-160	147	whole	y = 0.3912x - 0.9984	0.5542	14	<0.001
		capsule	y = 0.0424x + 0.9275	0.2052	14	NS

Effect of PO₂ on V̇O₂

Within each age category, V̇O₂ was plotted against PO₂ for the whole egg and for the capsules and linear regressions were fitted to the data (Fig. 4.3, Table 4.1). There was a weak trend of increasing V̇O₂ with PO₂, which was significant in the initial four age categories (11, 48, 70, 90 days) and in the final age category (147 days), however, only in the final age category was the slope strongly significant (p<0.001).
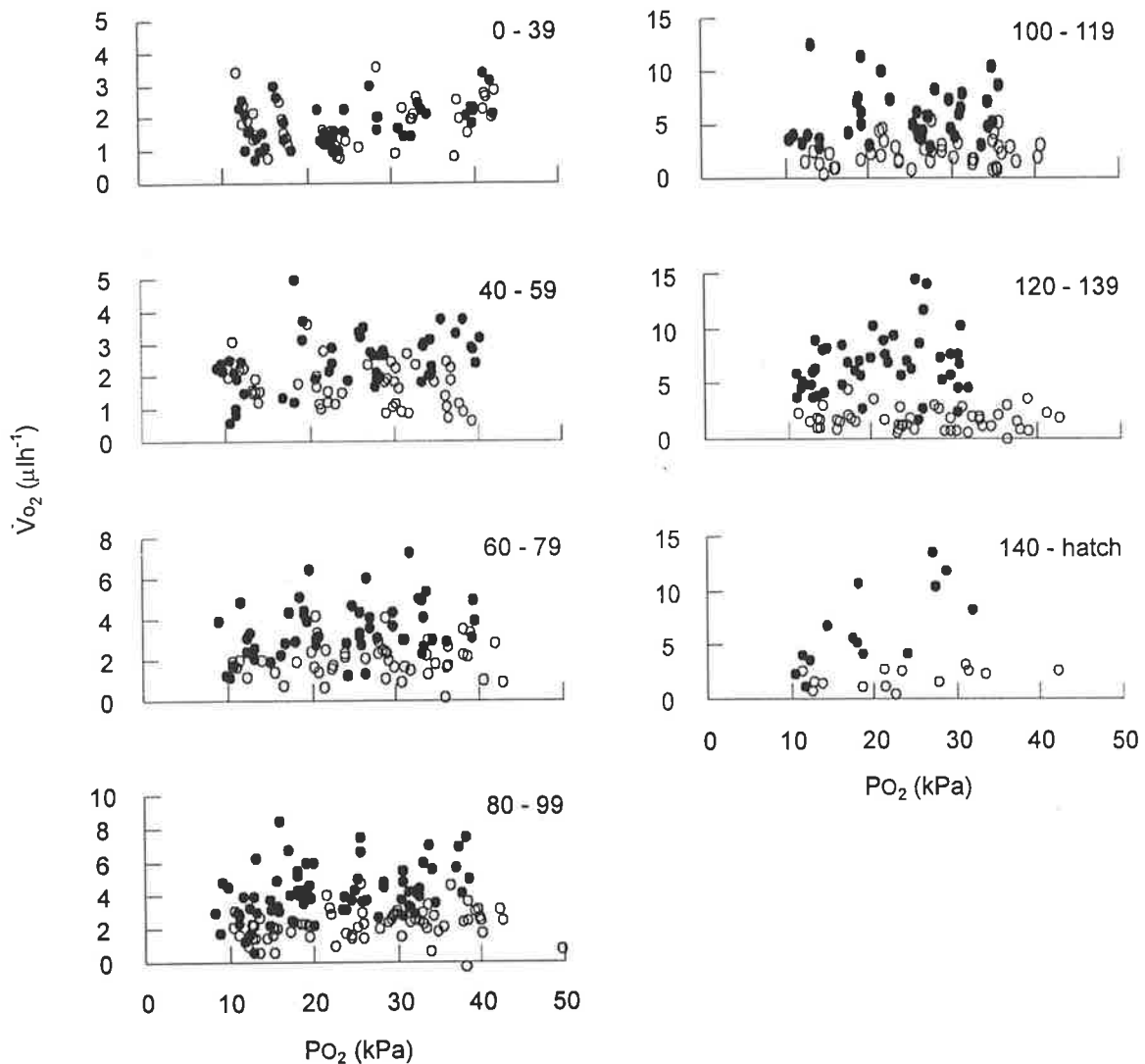
Figure 4.3: Whole egg (filled) and capsule (open) oxygen consumption (V̇O₂) as a function of chamber PO₂. Each point represents an average V̇O₂ for a single egg or capsule. The age category of the eggs is shown in the top right hand corner.

The hatchling $\dot{V}O_2$ was independent of PO_2 above 8 kPa, whereafter $\dot{V}O_2$ abruptly dropped to near 0 μl h^{-1}, indicating that hatchling $\dot{V}O_2$ was dependent on PO_2 (Fig. 4.4). Despite the subjection to hypoxia, the hatchlings recovered when returned to aerated seawater.
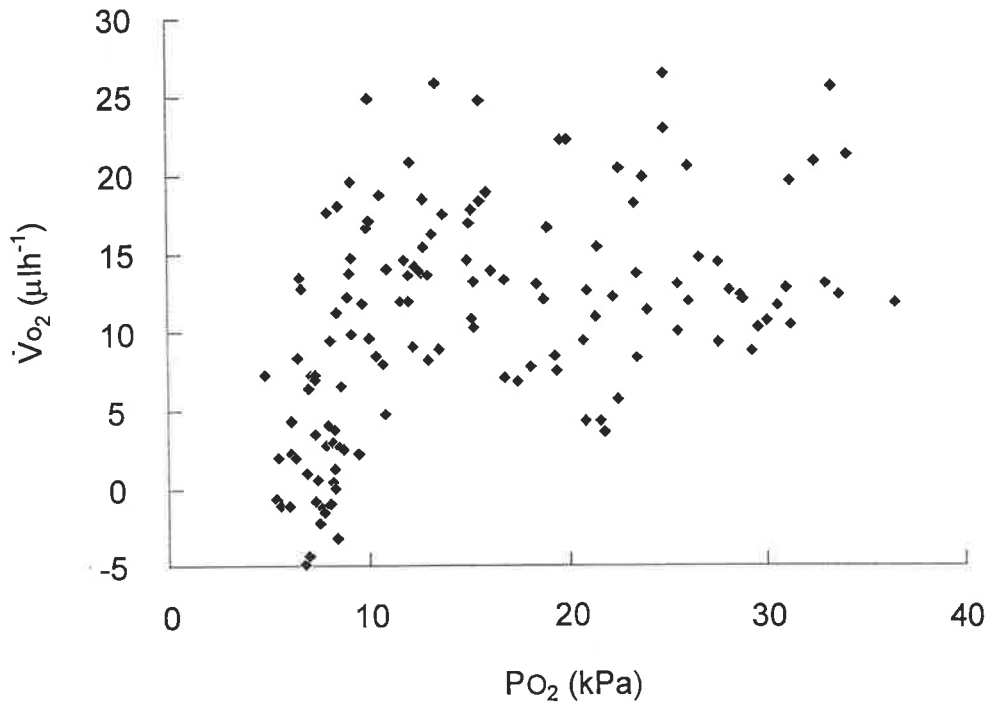
Figure 4.4: The oxygen consumption rate of hatchlings at 12°C in relation to ambient PO_2. Each point represents an individual measurement from 16 hatchlings.

Changes in $\dot{V}O_2$ throughout development

For each age category, the average $\dot{V}O_2$ was calculated over the PO_2 range for the whole eggs and the capsules, and the embryonic consumption calculated as the difference between the two. The consumption rate of the capsules remained relatively constant with age, whilst the whole egg $\dot{V}O_2$ (and therefore embryonic $\dot{V}O_2$) increased (Fig. 4.5). At the onset of development, the mean $\dot{V}O_2$ of the whole eggs (2.02 $\pm$ 0.12 (27) μl h^{-1}) was similar to that of the capsules (1.53 $\pm$ 0.12 (27) μl h^{-1}). The whole egg $\dot{V}O_2$ increased thereafter reaching a maximum of 7.36 $\pm$ 1.01 (12) μl h^{-1} by day 147. Consequently, the embryonic $\dot{V}O_2$ increased throughout development from near 0 to 5.47 $\pm$ 0.89 (12) μl h^{-1} by the final age category. Upon hatching the $\dot{V}O_2$ rate increased markedly, to reach 13.58 $\pm$ 0.69 (16) μl h^{-1} (Fig. 4.4).

Using the straight line relationship between capsule $\dot{V}O_2$ and age (Fig. 4.5), the total oxygen consumed per day could be determined, and then integrated over the developmental period. In 160 days the capsule consumed 6.93 ml O_2. The embryonic oxygen consumption was similarly determined by integration of the daily oxygen consumption determined from the polynomial equation describing embryonic $\dot{V}O_2$ and age (Fig. 4.5). However, an additional 32.5% was added to compensate for the increased $\dot{V}O_2$ with stirring. The polynomial equation provided a minimum embryonic $\dot{V}O_2$ at day 20 which was assumed to hold for the initial 20 days of development. The production from yolk to hatchling of S. apama over 160 days at 12°C, required 13.14 ml O_2 (assuming aerobic respiration).
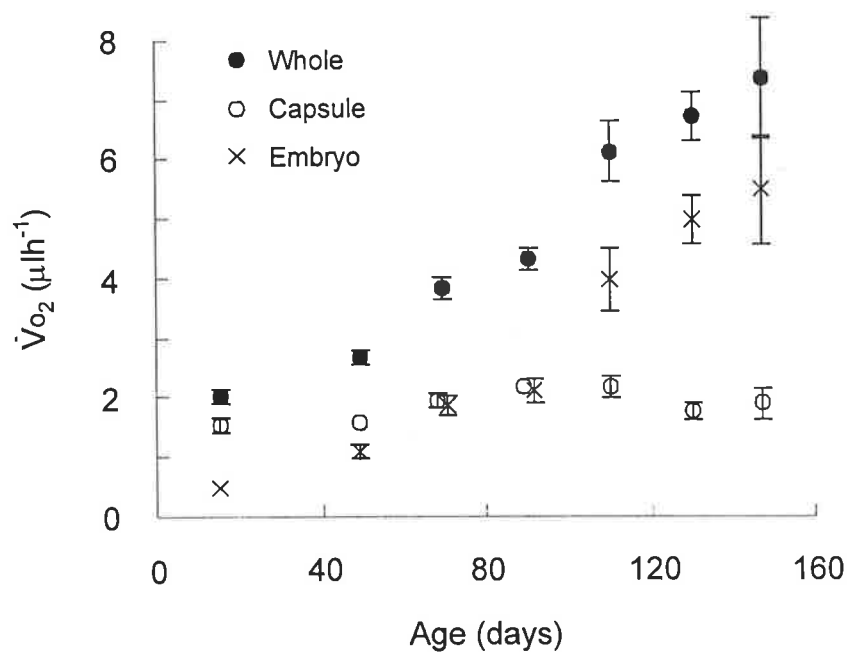
Figure 4.5: Rates of oxygen consumption of the whole egg, capsule only and embryos throughout development at 12°C. In total 312 eggs were measured with replicates for each point ranging from 12 to 65. Bars represent standard error of the mean. Equations to describe that data are: Whole egg $\dot{V}O_2 = -3 \times 10^{-6} \times age^3 + 0.0009 \times age^2 - 0.0256 \times age + 2.2338$, $r^2 = 0.9872$; Embryo $\dot{V}O_2 = -2 \times 10^{-6} \times age^3 + 0.0007 \times age^2 - 0.0246 \times age + 0.7667$, $r^2 = 0.979$; Capsule $\dot{V}O_2 = 0.0029 \times age + 1.6115$, $r^2 = 0.2623$.

Perivitelline Fluid PO₂

The PO_2 of the PVF decreased with increased age of the egg to approximately 110 days whereafter the PO_2 remained nearly constant at 5.85 ± 0.34 (5) kPa (Fig. 4.6).
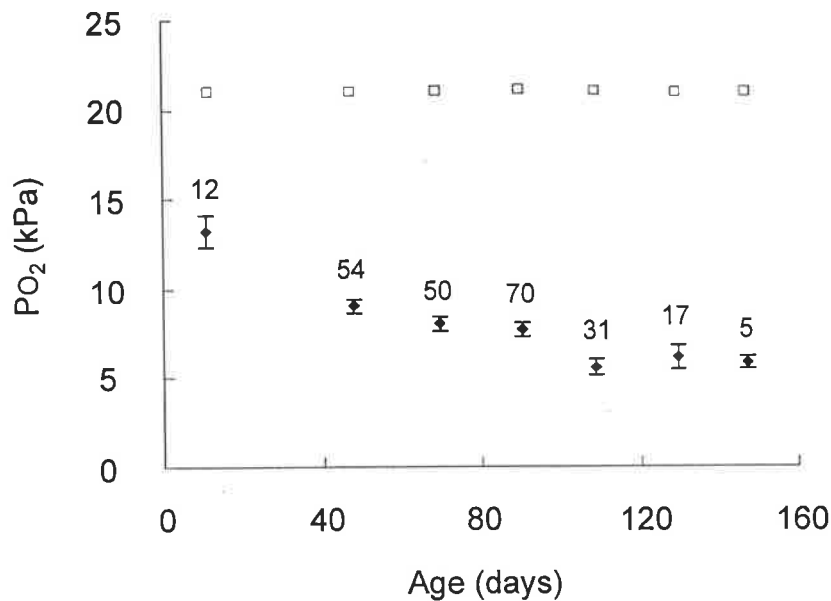
Figure 4.6: Partial pressure of the internal (diamonds) and external (squares) environments during development. The number of eggs measured (n) is shown above the points, bars represent standard error of the mean.

Oxygen conductance of the capsule

Capsule oxygen conductance (GO_2) was calculated from the embryonic $\dot{V}O_2$ (calculated as the difference between whole egg and capsule only $\dot{V}O_2$) and ΔPO_2 (equation 1). It increased gradually prior to 90 days of development, and then increased rapidly thereafter (Fig. 4.7), reaching 0.66 ± 0.26 (5) $\mu l \ h^{-1} \ kPa^{-1}$ by the final age category. By using the calculated GO_2 and measurements of capsule area and thickness (Chapter 2), Krogh's coefficient of diffusion (KO_2) through the capsule could be calculated over the developmental period. Averaging the values for KO_2 over the developmental period gave an estimate for KO_2 of $3.39 \times 10^{-8} \ cm^2 \ min^{-1} \ kPa^{-1}$, which is approximately 10% of pure water at 12°C (Table 4.4).
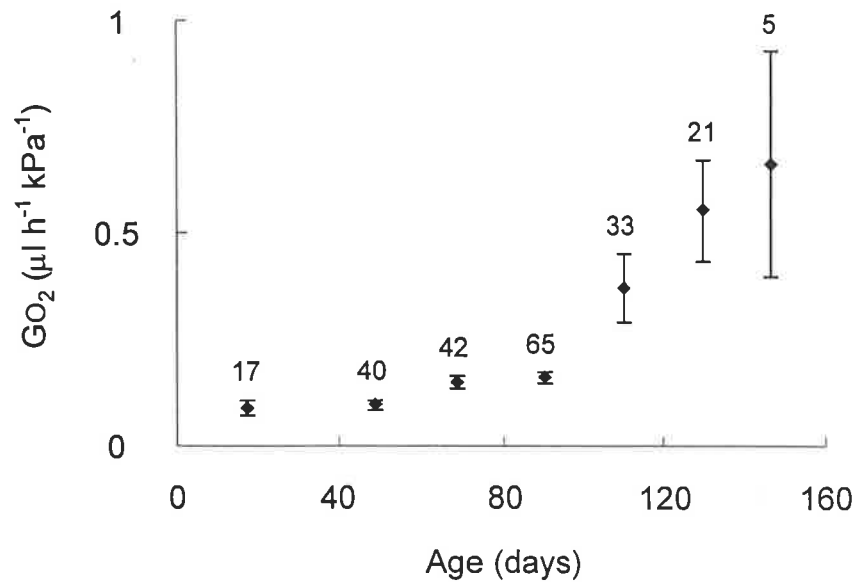
Figure 4.7: Oxygen conductance of the capsule of eggs during development at 12°C. The number of eggs measured (n) is shown above the points, bars represent the standard error of the mean.

Role of convection

Convection occurs very early in development by the beating of cilia on the yolk, which circulates the PVF in a posterior to anterior direction, drawing water across the mantle and head of the embryo and around the yolk. This circulates back along the chorion membrane to the anterior end of the egg (Fioroni 1990). Later in development, fluttering of the fins and contractions of the mantle generate convective currents. Mantle contractions draw water across the gills, the rate of which is dependent on the external environment.

For late stage eggs mantle contraction rate was positively correlated with temperature ($p<0.001$) and negatively correlated with PO_{2out} ($p<0.001$) (Fig. 4.8). Increasing the temperature from 15°C to 20°C resulted in a 100% increase in mantle contraction rate from 22 to 44 contractions min^{-1}, whilst the contraction rate at 12°C was less than half of the rate at 15°C. Decreasing the PO_{2out} to 70% of saturation resulted in an increase in contraction frequency from 29 to 36 contractions min^{-1}, whereas the mantle contraction rate was almost

undetectable at 54 kPa. Increased mantle contraction frequency was concurrent with obvious indentation of the ventral mantle indicating greater amplitude of contraction. In addition, the embryo often lifted itself and attached yolk from the floor of the capsule in brief spasms of activity. Prolonged activity during exposure to low PO_{2out} resulted in hatching on several occasions. In contrast, at low mantle contraction frequencies the embryo remained quiescent within the egg, contracting the mantle at irregular intervals.
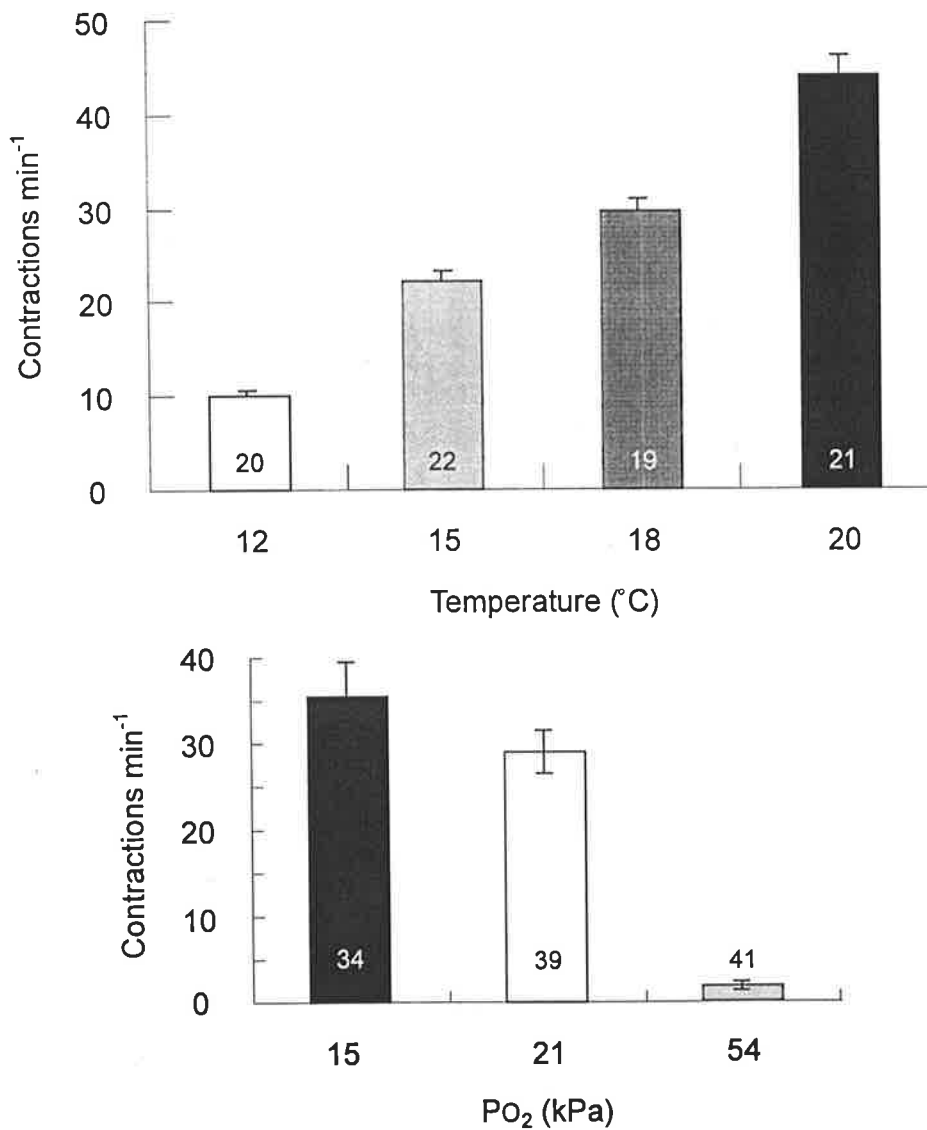
Figure 4.8: The effect of temperature (top), and partial pressure (bottom), on mantle contraction rate of late stage embryos. Bars represent standard error of the mean. The number of replicates is indicated at the base of each column.

Discussion

Embryonic oxygen consumption

The embryonic $\dot{V}O_2$ has been described by a 3rd order polynomial equation (Fig. 4.5), however this does not provide insights into the relationship between $\dot{V}O_2$ and embryonic development that can be easily compared between animal groups. By log transforming the data, linear equations can be fitted to the data to better interpret the relationships between $\dot{V}O_2$ and development. By log transforming the embryonic $\dot{V}O_2$ ($\mu l\ h^{-1}$) against age (days), a linear plot of the relationship could be determined (Fig. 4.9), and a power equation of the form $\dot{V}O_2 = a\ (age)^b$, generated for *S. apama* whereby;

$$\dot{V}O_2 = 0.021\ (age)^{1.08}$$

The metabolic rate increases in virtually direct proportion to the increase in age (developmental time) as indicated by the exponent (b) of 1.08. Near linear increases in $\dot{V}O_2$ with time are also evident in fish embryos, including turbot (*Scophthalmus maximus*), (Finn *et al.* 1995), medaka (*Oryzias latipes*), (Hishida and Nakano 1954), lumpsucker (*Cyclopterus lumpus*), (Davenport 1983), cod (*Gadus morhua*), (Davenport and Lonning 1980), and lemon sole (*Microstomus kitt*), (Ronnestad *et al.* 1992). The $\dot{V}O_2$ increases during development as the mass of metabolising tissue increases. The relationship between $\dot{V}O_2$ ($\mu l\ h^{-1}$) and dry mass of the embryo with internal yolk removed (g) can be analysed in a similar manner to $\dot{V}O_2$ and age, by regressing the logarithms to derive a linear relationship (Fig. 4.10), and log transforming the equation to arrive at a power equation;

$$\dot{V}O_2 = 29.980\ (mass)^{0.525}$$

The metabolic mass exponent (b) of 0.52 indicates the rate of increase in the $\dot{V}O_2$ declines as the embryo dry mass increases, resulting in a decline in dry mass specific metabolic rate throughout development. For a developing embryo, the observed $\dot{V}O_2$ is the sum of the

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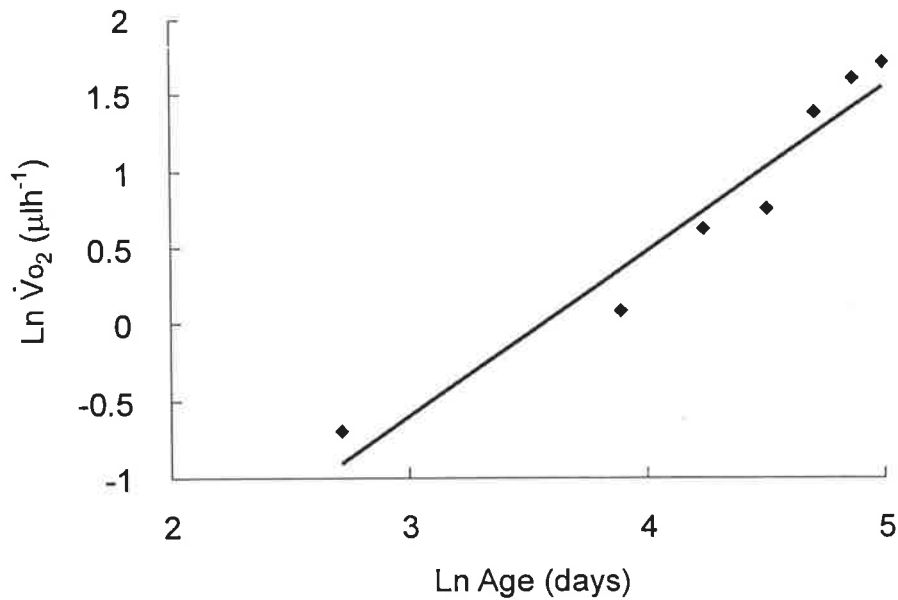
Figure 4.9: Relationship between natural logarithms of embryonic $\dot{V}O_2$ and age. The equation of the straight line is; Ln $(\dot{V}O_2)$ = - 3.85 + 1.08 × Ln (age), r^2 = 0.94. Log transforming provides the relationship $\dot{V}O_2$ = 0.021 × age$^{1.08}$, identifying a= 0.021 and b = 1.08.
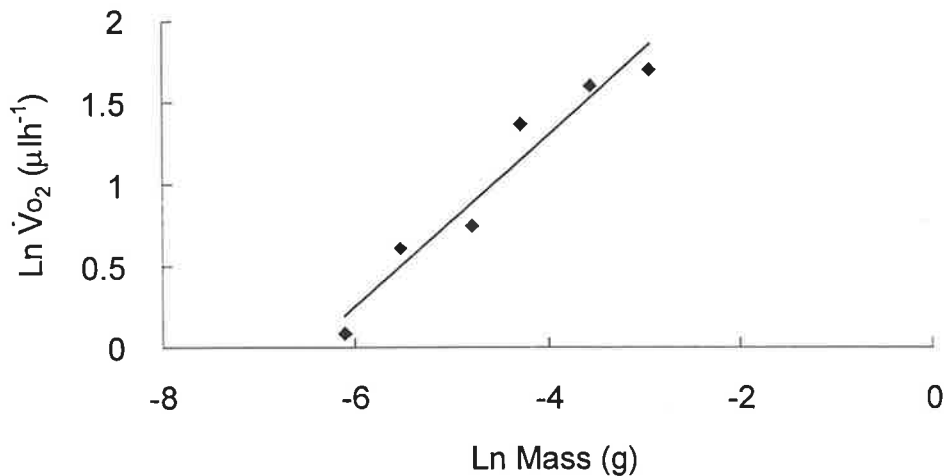
Figure 4.10: Relationship between natural logarithms of embryonic $\dot{V}O_2$ and mass (embryo - internal yolk). The equation of the straight line is Ln $(\dot{V}O_2)$ = 3.40 + 0.53 × Ln (mass), r^2 = 0.94. Log transforming provides the relationship $\dot{V}O_2$ = 29.980 × mass$^{0.525}$.

metabolic costs of the production of new tissue, the maintenance of existing tissue and the activity of the embryo (Chapter 5). For species with decreasing mass-specific metabolic rates, either the metabolic costs of maintenance must decrease as tissue mass increases and/or growth rate decreases. Variation in the mass-specific metabolic rate throughout development is expected as the costs of growth of different tissues vary according to tissue type and water content, and maintenance costs change with size and developmental maturity and functionality (Vleck and Bucher 1998). The mass-specific metabolic rate generally decreases as development proceeds, to the exponent of 0.8 in juvenile and adult fish (Winberg 1956), however it may approach or exceed unity for many species of fish larvae (Rombough 1988b). Conversely, metabolic mass exponents as low as 0.42 in tilapia fry (DeSilva *et al.* 1986), 0.55 in larval haddock (Lawrence 1978), and 0.65 in larval plaice (DeSilva and Tytler 1973) have been reported. Therefore the metabolic mass exponent of 0.52 for *S. apama* embryos is within the range reported for fish larvae. The metabolic mass exponent for *S. apama* was determined for the dry mass of the embryo excluding the internal yolk. Reducing the mass to that of the metabolising tissue results in a lower metabolic mass exponent in comparison to when the residual yolk is included ($\beta = 0.65$). This may explain the large difference between the value obtained for *S. apama* compared to fish larvae, where the distinction between metabolising tissue and yolk reserves is rarely taken into account. However, the low metabolic mass exponents may also be a result of limitation to $\dot{V}O_2$ by either the capsule or the conditions in the respirometer.

The $\dot{V}O_2$ of late stage *S. apama* embryos are comparable to those of stage 29 (Lemaire) *S. officinalis* embryos (Wolf *et al.* 1985) converted for 12°C (Withers 1992) (Table 4.2: row 3). Wolf *et al.* (1985) did not supply embryo masses, however values from Bouchaud (1991) (Table 4.2: row 1 and 2) were used for mass specific comparisons. Assuming the mass determined by Bouchaud (1991) are correct this provides a mass specific embryonic $\dot{V}O_2$ for *S. officinalis* more than double that for *S. apama* (Table 4.2: row 4 and 5). Mass specific $\dot{V}O_2$

decreases with increased body mass, therefore only a slightly greater mass specific metabolic rate in $S.$ $officinalis$ is expected. For $S.$ $officinalis$ of between 0.12 and 1500 g at 17°C, the mass specific $\dot{V}O_2$ decreases with increased body mass, whereby; $\dot{V}O_2 = 0.1373\ M^{-0.09}$ ml $g_{(wet)}^{-1}\ h^{-1}$ (Johansen $et\ al.$ 1982). If this equation is used to predict embryonic $\dot{V}O_2$ of the two species, the difference between the two species is much smaller (Table 4.2: row 6). Therefore, either the mass specific $\dot{V}O_2$ of $S.$ $officinalis$ is much greater than expected, or that of $S.$ $apama$ somewhat low. The increase in $\dot{V}O_2$ of hatchling $S.$ $apama$ (Table 4.2: row 7 and 8) reduces the difference in $\dot{V}O_2$ between the species to a value similar to that predicted by the equation. This suggests the $\dot{V}O_2$ of late stage $S.$ $apama$ embryos are lower than expected, and that the embryos are diffusion limited.

Table 4.2: Comparison between $S.$ $apama$ and $S.$ $officinalis$ masses and metabolic rates at 12°C for late stage embryos and hatchlings. * late stage embryo removed from capsule. Data for $S.$ $officinalis$ from Wolf $et\ al.$ (1985) and Bouchaud (1991), and equation from Johansen $et\ al.$ (1982).

	Parameter	Units	$S.$ $apama$	$S.$ $officinalis$
1	Total wet mass	(g)	0.496	0.250
2	Yolk free dry mass	(g)	0.052	0.029
3	Embryonic $\dot{V}O_2$	$(\mu l\ h^{-1})$	5.5	6.5
4	Embryonic $\dot{V}O_2$	$(\mu l\ g_{(dry)}^{-1}\ h^{-1})$	105.2	224.1
5	Embryonic $\dot{V}O_2$	$(\mu l\ g_{(wet)}^{-1}\ h^{-1})$	11.0	26.2
6	$\dot{V}O_2$ from equation $\dot{V}O_2 = 0.1373\ M^{-0.09}$	$(\mu l\ g_{(wet)}^{-1}\ h^{-1})$	96.4	102.6
7	Hatchling $\dot{V}O_2$	$(\mu l\ h^{-1})$	13.6	7.0*
8	Hatchling $\dot{V}O_2$	$(\mu l\ g_{(wet)}^{-1}\ h^{-1})$	27.4	28.2*

The $\dot{V}O_2$ of *S. apama* hatchlings (27.4 μl O_2 $g_{(wet)}^{-1}$ h^{-1}) compare with a average value of 166.2 μl O_2 $g_{(wet)}^{-1}$ h^{-1} calculated from a collection of consumption rates from various species of cephalopods (Table 4.3). However, the values vary from as low as 11.64 μl O_2 $g_{(wet)}^{-1}$ h^{-1} (Seibel *et al*. 1997) to 561.8 μl O_2 $g_{(wet)}^{-1}$ h^{-1} (DeMont and O'Dor 1984) and depends on temperature, size, maturity and activity. Values for *Sepia officinalis* of 50g or greater approximate 86 μl O_2 $g_{(wet)}^{-1}$ h^{-1}. The $\dot{V}O_2$ of *S. apama* hatchlings is 1/3 of the adult *Sepia officinalis* which is unexpected as a smaller individual is expected to have a greater mass specific metabolic rate (Johansen *et al*. 1982). In birds the embryonic metabolic rates are lower than what is predicted for similarly sized adults of the same species, whereas metabolic rate of the hatchlings is greater (in all but altricial birds) than that predicted from adult allometries (Vleck and Bucher 1998). However the interpretation of the hatchlings metabolic rate depends on the technique used to generate the equivalent adult size 'expected' rate. In cephalopods, metabolic rates are highly dependent on activity, which is often difficult to control in an experimental set up measuring $\dot{V}O_2$ (DeMont and O'Dor 1984). The compiled juvenile and adult $\dot{V}O_2$ are from different experimental set-ups and activity levels, making comparisons between life stages difficult.

Capsule oxygen consumption

Whilst the whole egg $\dot{V}O_2$ increased throughout development, the capsule $\dot{V}O_2$ remained relatively constant throughout development, suggesting the capsule is occupied by a stable population of bacteria. The bacteria are probably introduced to the capsule during deposition via the accessory nidamental gland of the female which has a duct opening in the mantle cavity near to the opening of the nidamental gland (Bloodgood 1977). The accessory nidamental gland changes colour from white to orange/dark red upon sexual maturation as a result of an abundance of pigmented bacteria in the tubules (Bloodgood 1977, Kaufman *et al*. 1998). In *Loligo opalescens* bacteria are present throughout the capsule as early as 6 hours after deposition, which further suggests a maternal origin (Biggs and Epel 1991). Barbieri *et al*. (1997) identified three major bacteria species isolated from the accessory

nidamental gland of *Loligo pealei*, at least one of which exhibited antifungal properties. Capsule bacteria act as symbionts, providing a means of defence against bacteria, fungus and predatory attack in eggs exposed to the environment for extended periods of time. The bacteria prevent colonisation and attack by pathogens by either their dense colonisation, by eliminating any resources for other potential pathogens, or by producing inhibitory or antibiotic compounds (Biggs and Epel 1991). Symbiotic bacteria have also been identified in the embryos and juveniles of the crustaceans, *Palaemon macrodactylus* and *Homarus americanus*, both of which brood the eggs externally (Gil-Turnes *et al.* 1989, Gil-Turnes and Fenicel 1992). Other means of defence include the incorporation of compounds acquired from the adults sponge diet and concentrated >10 times in the egg ribbons of *Hexabranchus sanguineus* to deter predators (Pawlik *et al.* 1988)), and the proposed chemical defences incorporated within the egg capsules of many pelagic lecithotrophic antarctic marine invertebrates eggs (McClintock and Baker 1997). The incorporation of defensive or protective mechanisms is necessary for eggs which have a long developmental duration and are therefore exposed to predators and pathogens for extended periods, however the production of protective capsular material is likely to incur some costs during egg production (Chapter 5).

Capsule $\dot{V}O_2$ represents a significant portion of the whole egg $\dot{V}O_2$ (Fig. 4.5), exemplifying the importance of measuring egg components separately. However, the impact of bacterial oxygen consumption is often ignored in the calculation of metabolic rates of organisms, despite it often being significant (Giorgio *et al.* 1997), resulting in erroneous measurements of metabolic rate (Dalla-Via 1983). The use of antibacterial agents is sometimes used in an attempt to eliminate bacterial growth, however they may have detrimental impacts on the organisms being measured. The treatment of eggs with penicillin and streptomycin to eliminate bacterial respiration has been used for *S. officinalis* eggs (Wolf *et al.* 1985), however the effect of the antibiotics was not compared with untreated eggs, furthermore the use of penicillin is futile in marine systems as it targets bacteria not abundant in seawater

(Dalla-Via 1983). Consideration of bacterial oxygen consumption is also necessary as it influences the diffusion of oxygen across the capsule and availability to the embryo. As oxygen diffuses across the capsule, bacterial consumption would reduce the amount of oxygen passing to the embryo. This would increase the likelihood of diffusion limited embryonic oxygen consumption during certain periods, such as approaching hatch, and if the PO_2 of the external environment were to decrease.

Effects of stirring on egg metabolic rate: Boundary layers

Field observations of *S. apama* eggs clustered together covered with fine debris indicate the eggs are not exposed to strong currents (Chapter 2). In addition, egg swelling of *Loligo vulgaris* was optimal in weak currents as opposed to strong ones (Jecklin 1934). Therefore, the $\dot{V}O_2$ measured in periodically stirred chambers (which was 67.5% of the constantly stirred rate) are reported because these conditions better represent natural conditions. The lower $\dot{V}O_2$ of whole *S. apama* eggs in periodically stirred chambers compared to constantly stirred chambers is suspected to result from the build-up of a boundary layer around the egg which would increase the diffusive distance for oxygen and reduce the rate of gas exchange. Giorgi (1984) measured PO_2 inside lingcod egg masses deposited in low currents at 16% saturation compared to 69% saturation at high current sites, and Pinder and Feder (1990) demonstrated a 55% increase in the resistance to oxygen uptake as a result of reducing water velocities from 5 cm s^{-1} to 0.1 cm s^{-1} for adult amphibians. These examples illustrate a significant effect of boundary layers inhibiting the exchange rate of materials with the environment. Seymour (1994, 1999) demonstrated that boundary layers have a marginal effect on the internal PO_2 of frog eggs if the perivitelline space is sufficiently large. By modelling changes in the internal and external radii of a typical frog egg, Seymour (1994) showed that PO_{2in} is far more sensitive to changes in the internal radius compared to changes in the external radius. By simplifying the shape of a *S. apama* egg to that of a sphere, the equation can be used to estimate the increase in thickness responsible for the lower $\dot{V}O_2$ of the egg in periodically stirred chambers. Assuming an egg approaching hatch has a diameter of 2.05

cm and thickness of 0.3 mm, a $\dot{V}O_2 = 11$ µl h^{-1} (with constant stirring), and KO_2 equivalent to 10% of water (3.39×10^{-8} cm^2 min^{-1} kPa^{-1}), the resultant ΔPO_2 is 12.7 kPa. An increase of 1.5% in the external radius (r_o), results in a 50% increase in the capsule thickness (X), equivalent to a boundary layer of 0.15 mm. This causes a reduction in $\dot{V}O_2$ to 7.39 µl h^{-1} which approaches the $\dot{V}O_2$ in chambers with periodic stirring. This illustrates that boundary layers have a large impact on embryonic $\dot{V}O_2$ when the external radius approaches the internal radius. Thus *S. apama* eggs could be very sensitive to fluctuations in current speed, and require deposition at sites with sufficient flow to minimise boundary layer formation. However, at Edithburgh, males preferentially selected dens with only one opening (Rowling 1994), which would minimise water flow across the eggs. It is probable that alternative factors such as the selection of secluded sites offering protection prevail over the selection of sites with optimal flow.

Effect of PO$_2$ on $\dot{V}O_2$

In *S. apama* embryos, there were positive trends of PO$_2$ on $\dot{V}O_2$, which were significant in the initial 100 days of development and again at the final age category. This identifies *S. apama* embryos as oxygen conformers during these periods. From 100 to 140 days, there was not a significant effect of PO$_2$ on $\dot{V}O_2$, therefore *S. apama* embryos can maintain their $\dot{V}O_2$ during these periods and are said to be oxygen regulators. The point at which the $\dot{V}O_2$ becomes dependent on the ambient PO$_2$ is the critical oxygen tension (P_{crit}) (Prosser and Brown 1962). The P_{crit} could not be identified at any age category in *S. apama* as the response to PO$_2$ was gradual. Alternatively, the PO$_2$ may not have been lowered sufficiently to effect a rapid change in $\dot{V}O_2$, identifying the P_{crit}. In adult *Sepia officinalis*, P_{crit} occurs at approximately 9.3 kPa, whereas the $\dot{V}O_2$ of hatchlings does not decline until a PO$_2 = 5.3$ kPa (Johansen *et al.* 1982). This suggests *S. apama* embryos may also be extremely tolerant of low PO$_2$, and that PO$_2$ was not lowered sufficiently to determine a P_{crit}.

Table 4.3: Metabolic rate (MR) of cephalopods. Metabolic rates were calculated at 12°C for comparison with *Sepia apama* embryos and hatchlings using the equation from Withers (1992): $R_2 = R_1 \times 10^{(\log Q_{10} \times (T_2-T_1)/10)}$, where R_1 = MR at temperature T_1, R_2 = MR at temperature T_2, Q_{10} = 2.3 (average from Boyle (1987)).

Species	Temp (°C)	n	wet mass (g)	MR (μmol O_2 g_{wet}^{-1} h^{-1})	MR (μl O_2 g^{-1} h^{-1})	MR @12°C (μl O_2 g^{-1} h^{-1})	Source
Abraliopsis felis	5	1	0.99	3.4	77.1	138.0	(Seibel *et al.* 1997)
Cranchia scabra	5	1	35.39	0.3	6.5	11.6	(Seibel *et al.* 1997)
Leachia dislocata	5	1	3.27	0.7	15.7	28.1	(Seibel *et al.* 1997)
Ocythoe tuberculata	5	1	1.21	4.2	93.4	167.3	(Seibel *et al.* 1997)
Octopus rubescens	5	2	0.06	7.5	167.6	300.2	(Seibel *et al.* 1997)
Abraliopsis pacificus	5	5	1.22	2.4	53.5	95.9	(Seibel *et al.* 1997)
Enoploteuthis higginsi	5	1	6.47	5.6	125.2	224.3	(Seibel *et al.* 1997)
Pterygioteuthis microlampas	5	1	0.13	6.5	144.7	259.2	(Seibel *et al.* 1997)
Ctenopteryx siculus	5	1	4.24	2.8	62.9	112.8	(Seibel *et al.* 1997)
Leachia pacifica	3.5	3	1.52	0.8	18.1	36.8	(Seibel *et al.* 1997)
Megalocranchia fisheri	5	1	47.90	0.4	8.7	15.7	(Seibel *et al.* 1997)
Cranchia scabra	5	1	6.39	0.4	9.0	16.1	(Seibel *et al.* 1997)
Illex illecebrosus	5			14.0	313.6	561.8	(DeMont and O'Dor 1984)
'composite' squid	14	-	41	11.4	255.4	215.0	(O'Dor and Wells 1987)
Illex illecebrosus	12.9		309	13.8	309.1	285.8	(O'Dor and Wells 1987)
Loligo opalescens	15		0.03	30.8	689.9	537.4	(Hurley 1976)

Sepioteuthis sepioidea	28		0.1	28.6	640.6	168.8	(LaRoe 1971)
Sepia officinalis	24		50	13.2	295.7	109.0	(Montuori 1913)
	16		3	6.2	138.9	98.9	(Raffy and Ricart 1939)
	17		50	4.6	103.0	68.6	(Johansen *et al.* 1982)
	17		1000	3.6	80.6	52.8	(Johansen *et al.* 1982)
40 day-old juvenile	20	45			238.0	122.2	(Wolf *et al.* 1985)
late stage embryo (removed from shell)	20	8		13.7 µl animal^{-1} h^{-1}		28.2	(Wolf *et al.* 1985)
Sepia apama hatchling	**12**	**16**	**0.50**	**13.6 µl animal^{-1} h^{-1}**		**27.4**	**this study**

Embryonic $\dot{V}O_2$ increases during development despite the gradual decrease in PO_{2in} to 5.8 kPa by 60-70% of development (Fig. 4.6). The PO_{2in} decreases owing to the changes in capsule GO_2 being insufficient to match the increasing metabolic demands of the embryo. The GO_2 increases only slightly (Fig. 4.7) because the increases in effective surface area and decreases in capsule thickness occur very gradually during this period (Chapter 2). As a result the $\dot{V}O_2$ is dependent on PO_2 during the first 100 days of development (Table 4.1). In the latter 30-40% of development the PO_2 stabilises (Fig. 4.6) indicating that changes in GO_2 match the increases in $\dot{V}O_2$. The morphological characteristics of the egg change more rapidly (Chapter 2), enabling capsule GO_2 to increase at a rate sufficient to match the increase in $\dot{V}O_2$ (Fig. 4.7). Consequently, the $\dot{V}O_2$ is independent of PO_2 during this latter period, at least until very late in development. However, as the embryo approaches hatching stage the high $\dot{V}O_2$ is likely to be limited by diffusion through the capsule despite the high GO_2. Three lines of evidence support this statement.

Firstly, the late stage embryonic $\dot{V}O_2$ increases with increased PO_{2out}, suggesting that the capacity of the embryo to utilise oxygen was exceeding the supply by diffusion (Fig. 4.3).

Secondly, the oxygen consumption rate of the hatchlings almost doubled to reach 13.6 μl h^{-1} in resting individuals in comparison to the late stage embryonic $\dot{V}O_2$ of 5.5 μl h^{-1}. Post hatching increases in $\dot{V}O_2$ have been reported in teleosts (Eldridge *et al.* 1977, Davenport and Lonning 1980, Cetta and Capuzzo 1982, Davenport 1983, Solberg and Tilseth 1984, Finn *et al.* 1995), amphibians (Seymour and Bradford 1995) and birds (Vleck and Bucher 1998). The magnitude of increase is suggested to relate directly to the capsule resistance to gas exchange. The thin (0.7μm) egg capsules of cod offer little resistance and subsequently $\dot{V}O_2$ increases by only 40-60% (Davenport and Lonning 1980) in comparison to a 10-fold increase measured in Pacific herring (Eldridge *et al.* 1977). If the capsule does not impede

oxygen exchange no change in $\dot{V}O_2$ would be expected, as is observed in pigfish (Robertson 1974) and carp (Kamler 1976). Similarly there was little effect on $\dot{V}O_2$ following removal of the capsule in late stage *S. officinalis* embryos (Wolf *et al.* 1985). This is contrary to the results obtained for *S. apama* and suggests that *S. apama* are diffusion limited approaching hatch.

Thirdly, hatchling $\dot{V}O_2$ became dependent on PO_2 below 8 kPa (Fig. 4.4), which is 2.2 kPa greater than the PO_{2in} of the PVF of late stage eggs. The P_{crit} of *S. apama* hatchlings compares with a P_{crit} of 11 kPa (4 mg O_2 l^{-1}), determined for 40-day old *S. officinalis* juveniles at 20°C (Wolf *et al.* 1985) and of 5.3 kPa for hatchlings of 120-150 mg at 17°C (Johansen *et al.* 1982). The P_{crit} has been shown to decrease by 2-3 mg O_2 l^{-1} immediately following hatching in steelhead and atlantic salmon as a consequence of the removal of the capsule inhibiting gas exchange (Hayes *et al.* 1951, Rombough 1988a). A decrease in P_{crit} following hatching must result from the capsule impeding oxygen exchange as any activity associated with hatching would result in increased metabolic rates and therefore greater P_{crit}. Therefore, in *S. apama* a PO_{2in} lower than the P_{crit} of the hatchlings again suggests the embryo experiences diffusion limitation near to hatch.

Capsule KO_2

The calculated KO_2 for *S. apama* egg capsules approximates 10% of water, comparable to the values reported for atlantic salmon and dogfish eggs (Table 4.4). The diffusivity (DO_2) of atlantic salmon correlates to the proportion of pores in the egg capsule, suggesting that diffusion occurs through pores rather than the structural matrix in fish eggs (Wickett 1975). The leathery egg case of dogfish is structurally very different to that of atlantic salmon eggs yet a similarly low value for DO_2 was measured. Conversely the KO_2 of frog egg jelly is approximately 75% of water (Burggren 1985, Seymour 1994), whilst the KO_2 of egg masses of a species of gastropod mollusc approach that of water (Cohen and Strathmann 1996)

(Table 4.4). Frog egg jelly constitutes 98% water (Beattie 1980), whereas the egg capsules of *S. apama* contain 90% water (Chapter 2). If the diffusivity of oxygen in artificial gels decreases as the polymer concentration increases (Sato and Toda, 1983, Rennenberg *et al.*, 1988), jelly with a high water content should have a high diffusivity and consequently a high KO_2 (provided that βO_2 remains the same). The comparatively low water content of *S. apama* egg capsules combined with the expected low KO_2 through the chorion, may result in the low calculated KO_2.

Table 4.4: Estimates of diffusivity (DO_2) and Krogh's coefficient ($KO_2 = DO_2 \times \beta O_2$) in various media. *conversion assuming a capacitance coefficient (βO_2) equal to that of water at 15°C (3.38×10^{-4} ml O_2 cm^{-3} kPa^{-1} (Dejours 1981)).

Medium	Diffusivity (DO_2) [$\times 10^{-3}$ cm^2 min^{-1}]	Kroghs coefficient (KO_2) [$\times 10^{-7}$ cm^2 min^{-1} kPa^{-1}]	Reference
Water	1.2	4.05*	(Horne 1969)
	1.56	5.27	(Wickett 1975)
		3.15	(Burggren 1985)
		3.6	(Ultsch and Gros 1979)
Gelatine 15%		2.76	(Krogh 1919)
Frog egg jelly		2.38	(Burggren 1985)
		2.34-3.25	(Seymour 1994)
Gastropod egg mass	1.05	3.55*	(Cohen and Strathmann 1996)
Dogfish egg case	0.177	0.598*	(Diez and Davenport 1987)
Atlantic salmon egg capsule	0.108	0.365*	(Wickett 1975)
Cuttlefish egg capsule		**0.339**	**this study**

Role of convection

In *S. apama* the importance of convection for gas exchange is evident by the increase in mantle contraction frequency when the egg is exposed to decreased environmental PO_2 (Fig. 4.8). The embryo attempts to maintain its $\dot{V}O_2$ by increasing the flow of water past the gills, increasing both amplitude and frequency of mantle contractions. Ventilation of the skin is probably also important, as oxygen consumption via this organ in some octopods represents 41% of the total consumed (Madan and Wells 1996). Conversely, mantle contraction frequency decreases upon exposure to increased environmental PO_2. The ability of the developing embryo to stir this medium and create convective transport within the egg occurs in unison with the absorption of water to produce and increase the PVF volume (Kress 1975, Strathmann and Chaffee 1984). In fish eggs, P_{crit} values increase rapidly during early development in relation to metabolic rate until the embryo begins to move and stir the PVF (Rombough 1988a). Thereafter, P_{crit} values increase more gradually and in proportion to the metabolic rate. Exposure to increased temperature results in increased frequency of trunk flexures and fin flutter in atlantic salmon embryos (Peterson and Martin-Robichaud 1983), increased ventilation frequency in dog fish embryos (Thomason *et al.* 1996), and increased PVF velocities adjacent to the body wall of frog embryos (Burggren 1985). In the absence of this convective stirring the PVF would create an enormous barrier to oxygen diffusion to the embryo. Instead, convective currents increase the PO_2 at the embryo surface and decrease the PO_2 immediately under the capsule, thereby increasing the PO_2 gradient across the egg, effectively accelerating or enhancing the overall diffusion of oxygen to the embryo (Reznichenko *et al.* 1977, Hunter and Vogel 1986, Boletzky 1987a).

Whilst convection accelerates gas exchange, it also requires energy, thereby increasing embryonic $\dot{V}O_2$. Whether the increased $\dot{V}O_2$ is compensated for by enhanced gas exchange associated with convection has rarely been investigated. Strathmann and Strathmann (1995) found that the higher metabolic demands of the ciliated stage of three species of

opisthobranch gastropod egg masses resulted in the pH of the jelly declining as a result of carbon dioxide accumulation within the jelly and capsule. This indicates that the greater $\dot{V}O_2$ associated with stirring generated waste products at a rate exceeding their diffusion across the capsule, despite the gas exchange rate increasing due to convection. In embryonic skates, the late stage $\dot{V}O_2$ exceeds the rate of oxygen diffusion across the capsule (in the absence of ventilation), necessitating ventilation by tail beating to meet the metabolic demand (Leonard et al. 1999). Tail beating represents a 53-81% increase in the standard metabolic rate, but is fundamental for the delivery of sufficient oxygen to the developing embryo (Leonard et al. 1999). It appears that diffusion is insufficient to meet the increasing metabolic demands of embryos and alternative systems are necessary to aid in gas exchange, despite these systems increasing the $\dot{V}O_2$. Marthy et al. (1976) proposed that the PVF of cephalopod eggs contains a tranquillising substance that maintains the embryo in a quiescent state, preventing the wastage of energy through increased respiration associated with movement, and hindering premature hatching. Restricting movement to mantle flexures would reduce the $\dot{V}O_2$ and hence energy expenditure.

Consequences of diffusive limitations on egg design

The metabolic rate of *S. apama* embryos is restricted late in development despite the high capsule GO_2, the inherently low metabolic rate and consequently the long developmental duration, all of which relieve the pressure for diffusive supply of oxygen throughout development. The long developmental duration also results from eggs being laid in autumn, and consequently incubating throughout winter and spring in water temperatures that suppress metabolism. Eventually however, the GO_2 is insufficient to meet the respiratory demands of the embryo, leading to diffusion limitation as in many amphibian (Seymour and Bradford 1995) and bird eggs (Vleck and Hoyt 1991). *S. apama* eggs therefore represent the upper boundary of egg size under natural conditions, and alternative solutions would have to be contrived to enable any further increase in egg size.

The egg size of some of the incirrate octopods (Fig. 1.3a-c) approach or exceed that of *S. apama* (Table 4.5 and 4.6), however diffusive limitations are circumvented because the eggs do not have a capsule but are enclosed solely within the chorion membrane, thus minimising the diffusive barrier to the thickness of the chorion. However, since the chorion does not expand to the same degree as in jelly encapsulated eggs (Boletzky 1998), the surface area for oxygen uptake is limited and GO_2 is constrained. To compensate, the mother broods the eggs, maintaining them free of debris and ventilating them by flushing water from her funnel. Despite being ventilated, the large octopod eggs are also likely to experience diffusive limited oxygen consumption approaching hatch, when the $\dot{V}O_2$ is greatest. Evidence of egg brooding in teuthoid squids by the female carrying the egg mass around in her arms has been provided (Okutani 1983), however whether this confers any gas exchange advantage to the eggs is unknown.

The cirrate octopods produce even larger eggs than the incirrate octopods (Table 4.5 and 4.6). These eggs are not brooded, but are enclosed within a capsule of variable thickness (Fig. 1.3d and e) in addition to a tough outer shell, which provides protection throughout the long developmental duration. Diffusion is likely to be impeded through this outer shell in comparison to the gelatinous capsules of the decapods. However, cirrate eggs develop at very cold temperatures and consequently have a long developmental duration and are expected to have low rates of oxygen consumption, which would minimise the likelihood of diffusive limitation.

The largest eggs belong to *Nautilus* and consist of an ovum surrounded by the chorion and some gelatinous material, wrapped in a doubled layered capsule (Table 4.6). Channels formed by the pleated outer wall enable seawater to infiltrate between the capsule layers (Boletzky 1989) increasing the PO_2 difference between the embryo and its environment. Similar mechanisms to increase the PO_2 difference between the embryo and its environment occur in the much larger egg capsules of oviparous elasmobranches which become partially

open to seawater in the latter 2/3 of the embryonic period, which enables ventilation of the embryo (Diez and Davenport 1987, Leonard *et al.* 1999). The incorporation of ventilation mechanisms decreases the diffusive distance for oxygen and increases the PO_2 difference between the embryo and its environment, enhancing gas exchange and enabling greater $\dot{V}O_2$ and hence egg sizes. Since no *Nautilus* eggs have been found in the field, the incubation temperature is unknown, however, hatchling *Nautilus belauensis* have been captured at 100 m depth concurrent with the estimated depth of egg deposition from oxygen isotope studies corresponding to temperatures of 16-20°C (Ward 1987).

For eggs incorporated within egg masses, the size constraints are more severe. In many gelatinous mollusc eggs masses, centrally located embryos experience low oxygen tensions which may approach or exceed the limit of the oxygen supply required for development (Chaffee and Strathmann 1984, Booth 1995, Cohen and Strathmann 1996). This situation is relieved by the incorporation of pores or channels throughout the egg mass that permit interstitial water flow and minimise the diffusive distance for oxygen, as is the situation in some amphibian egg masses (Pinder and Feder 1990, Seymour *et al.* 1991, Seymour and Bradford 1995). However, if insufficient oxygen diffuses to the centrally located embryos, oxygen consumption is limited and may result in development being extended or temporarily arrested (Booth 1995, Strathmann and Strathmann 1995). Asynchronous development results in the peripherally located embryos hatching earlier, thus disintegrating the outer layers of the jelly mass and reducing the diffusive distance to the centrally located embryos, which are then permitted to develop further. The eggs incorporated into such gelatinous egg masses are typically less than 0.2 mm diameter (Strathmann 1987, Smith *et al.* 1989), and correspondingly have low metabolic rates and often hatch at pre-metamorphosed stages. In cephalopods, only eggs less than 1 mm diameter, such as the eggs of *Illex illecebrosus* and *Todarodes pacificus* are incorporated within egg masses (Table 4.6). These masses may exceed 30 cm in diameter, suggesting that asynchronous development is likely. The amorphous jelly secretion of these egg masses may easily disintegrate when peripherally

located embryos hatch, enabling the more centrally located embryos to develop. As egg size becomes larger, changes in egg mass design occur which minimise the diffusive distance for oxygen. Eggs are positioned in the outer layers of the egg capsule in *Dosidicus gigus* or within in a single mucous layer in *Gonatus fabricii* (Table 4.6). Interestingly, the amorphous egg masses tend to float freely rather than being attached to the substrata. This exposes the egg mass to the light which may enable photosynthesis of organisms within the jelly. This would increase the oxygen content within the capsule facilitating the supply of oxygen to eggs within the mass during daylight, but would increase the oxygen demands of the egg mass during darkness, as is the case in some amphibian (Pinder and Friet 1994) and gastropod egg masses (Cohen and Strathmann 1996).

In *Sepioteuthis* spp. several eggs are encased within a single strand, then collectively attached in clusters to the substratum (Fig. 1.2a and c). This strategy increases the surface area for gas exchange per egg provided that the strands are not too densely packed. Dense layered clusters would be likely to approach the structural design constraints of egg masses. Poor oxygenation of layered egg masses is suggested to be the cause of slowed embryonic development of eggs within the egg clusters of *Sepiola* and *Sepietta* species (Boletzky 1986) (Fig. 1.2f). A similar situation would likely result in dense clusters of *S. apama* eggs. Late in development the eggs contact one another reducing the surface area exposed to seawater, exacerbating the problems associated with gas exchange. In layered clusters of eggs the surface area for gas exchange would be further reduced, possibly resulting in hypoxic conditions for late stage embryos. Spaces between *S. apama* egg clusters would facilitate gas exchange, provided that water flow at the deposition site was sufficient (Chapter 2). Water flow around eggs was shown to affect the swelling (and mass increase) of *Loligo vulgaris* egg capsules (Jecklin 1934). Optimal mass increases occurred in weakly flowing water, whereas mass increases were lower in unstirred water and lowest in strongly flowing water. This implies that normal development (optimal egg swelling) is dependent on suitable water currents. In *Loligo opalescens* spawning grounds littered with egg fingers

covering a 12 m area over 1 m thick have been reported (Hixon 1983). It is likely that only the embryos near the surface of the spawning mass would receive sufficient oxygen for survival. However, the creation of a large spawning area may increase the survival and success of the juveniles in some other manner, compensating for losses resulting from inadequate oxygenation of the spawning mass.

In summary, *Sepia apama* eggs are diffusion limited approaching hatch owing to their large size and consequently high metabolic demands, which are eventually unmatched by increases in capsule GO_2. Relative to the size of the embryo, embryonic metabolism is suppressed owing to the low incubation temperatures and inherent metabolic depression which assuage the likelihood of diffusive limitation. Diffusive limitation is also mitigated by various means in the eggs or egg masses of other cephalopods. The depositional environment is important in ventilating eggs to maximise the PO_2 difference between the egg and its environment to facilitate gas exchange. However, all eggs approaching hatch are likely to experience diffusive limitation resulting from low PO_{2in}. The low PO_{2in}, has been suggested to stimulate the hatching response in many organisms (Dimichele and Taylor 1980, Petranka *et al.* 1982, Bradford and Seymour 1988), enabling the embryo to escape the confines of the oxygen limited environment.

Table 4.5: Summary of estimated volume of ovums of cephalopod species, listed from smallest to largest. Volumes are determined assuming ovums are spherical when only one dimension was provided, and using the formula $V = \frac{4}{3}\Pi \times r_l^2 \times r_d$, where r_l is largest radius and r_d is smallest radius, when both diameter and length were provided (Table 4.6). The ovum size of the cirrates and octopods were estimated assuming the ovum size = egg size.

Species	Teuthoidia	Sepioidea & Sepioliodea	Octopoda (incirrata)	Cirroctopoda (cirrata)
Todarodes pacificus	0.245			
Illex illecebrosus	0.318			
Idiosepius spp.		0.524		
Dosidicus gigas	0.524			
Thysanoteuthis rhombus	0.524			
Loligo peallii	1.340			
Octopus vulgaris*			2.094	
Spirula spirula		2.145		
Octopus tetricus*			2.352	
Argonauta spp.			2.618	
Octopus defilippi*			3.054	
Loligo opalescens	3.844			
Lolliguncula brevis	4.055			
Euprymna scolopes		4.156		
Sepiola robusta		4.562		
Robsonella australis*			5.964	
Loligo vulgaris	6.545			
Octopus burryi*			6.795	
Sepietta oweniana		8.181		
Loligo forbesi	10.187			
Octopus cyanea*			14.137	
Octopus bimaculatus*			20.944	
Octopus rubescens*			22.449	
Sepioteuthis australis	53.852			
Octopus fitchi^			65.450	
Octopus maorum^			65.450	
Eledone cirrhosa^			73.631	
Octopus joubini^			76.969	
Sepia officinalis		153.938		
Octopus dofleini^			179.594	
Octopus maya#			247.086	
Octopus briareus#			339.292	
Stauroteuthis syrtensis				380.132
Opisthoteuthis spp.				380.132
Eledone moschata			461.814	
Cirroteuthis muelleri				606.131
Sepia apama		**633.554**		
Cirrothauma murrayi				923.628
Octopus pallidus#			1150.346	
Grimpoteuthis antarcticus				1608.494

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Table 4.6: Cephalopod egg characteristics, listed according to species classification and then egg or ovum size. *small egg species with long duration planktonic larvae; ^ intermediate egg species with short duration planktonic or benthic hatchlings; # large egg species with benthic young. 'Egg size' includes the capsule, whereas 'ovum size' refers only to the yolk dimensions.

Species	Classification	egg type	egg size (mm)	ovum/ egg	ovum size	hatchling size (mm)	adult ML (cm)	duration of development	reference
Nautilus macromphalus	Nautiloidea	Pleated double walled capsules	36.8 x 24.8	1		25 mm across shell			(Ward 1987)
Vampyroteuthis infernalis	Vampyromorpha	Spherical - no jelly coat			3.8				(Hochberg and Nixon 1992)
Cirroteuthis muelleri	Cirrata	Tough ovoid capsules	10-11	1		11			(Hochberg *et al.* 1992)
Stauroteuthis syrtensis	Cirrata	Tough ovoid capsules	11 x 6	1					(Hochberg *et al.* 1992)
Opisthoteuthis spp.	Cirrata	Tough ovoid capsules	11 x 6	1					(Hochberg *et al.* 1992)
Cirrothauma murrayi	Cirrata	Tough ovoid capsules	14 x 0.9	1		10-15			(Hochberg *et al.* 1992)
Grimpoteuthis antarcticus	Cirrata	Tough ovoid capsules	16 x 12	1					(Hochberg *et al.* 1992)
Argonauta spp.	Octopoda	Calcified brood shell with air bubble		1	2-3, 0.8		25 cm diam		(Zeidler and Norris 1989), (Boletzky 1992)
*Octopus defilippi**	Octopoda	Individual capsules		1	1.5-2.1	1.3-1.5			(Hochberg *et al.* 1992)
*Octopus vulgaris**	Octopoda	String of stalked ovate capsules		1	2 x 1	1.72	1-1.5kg	25°C 22-25d, 13°C 125d	(Mangold 1983b)
*Robsonella australis**	Octopoda	Individual capsules		1	2.2-2.3	2.2-2.3			(Hochberg *et al.* 1992)

Species	Order	Description			Size				Reference
*Octopus burryi**	Octopoda	Individual capsules		1	2.2-2.5	1.5			(Hochberg *et al.* 1992)
*Octopus tetricus**	Octopoda	Eggs in string (1000-1500/string)		1	2.4-0.9	5	1-2kg	360d	(Joll 1983)
*Octopus bimaculatus**	Octopoda	Individual capsules		1	2.5 x 4	2.6			(Hochberg *et al.* 1992)
*Octopus cyanea**	Octopoda	String of stalked capsules (600-1200/string)		1	3	3	5-6kg	20-27°C 20-30d	(Van-Heukelem 1983a)
*Octopus rubescens**	Octopoda	Individual capsules		1	3-4	1.7-2			(Hochberg *et al.* 1992)
*Octopus fitchi^*	Octopoda	Individual capsules		1	4-6	2			(Hochberg *et al.* 1992)
*Octopus maorum^*	Octopoda	Individual capsules		1	4-6	4.3-4.5			(Hochberg *et al.* 1992)
*Octopus joubini^*	Octopoda	Stalked ovate capsules		1	6-8 x 2-4	5.5 (40mg)	2.2-4.2	25°C 35-40d	(Hanlon 1983b)
*Octopus dofleini^*	Octopoda	Eggs in strands (252/strand)		1	6-8	3.4	50+kg	160d - 7mths	(Hartwick 1983)
*Eledone cirrhosa^*	Octopoda	String of stalked ovate capsules		1	7.5 x 2.5	3	0.3-0.7 kg	16°C 100d	(Boyle 1983)
Octopus maya#	Octopoda	Eggs in string		1	11 x 3.9	93 mg	4kg	24-26°C 45d	(Van-Heukelem 1983b)
Octopus briareus#	Octopoda	Cluster of stalked capsules (25/cluster)		1	10-14 x 4-5	5.5 (95mg)	4.5-12	30°C 36d, 19-25°C 50-80d	(Hanlon 1983a)
Octopus pallidus#	Octopoda	Mass of tear shaped capsules		1	12-14		14		(Zeidler and Norris 1989)
Eledone moschata	Octopoda	Clusters of ovate capsules (3-10/cluster)		1	12-16 x4-5	10-12	0.5kg	10-22°C 180d	(Mangold 1983a)
Idiosepius spp.	Sepioidea	No capsule coat		1	1		1-1.5		(Boletzky 1998)
Spirula spirula	Sepioidea				1.5-1.7	1.5	5		(Zeidler and Norris 1989)
Euprymna	Sepioidea	Flat mass of	4	1	2.1x1.8	1.5	3.5		(Singley 1983)

scolopes									
		spherical capsules							
Sepiola robusta	Sepioidea	Flat mass of spherical capsules	3.5-4.5	1	2.2 x 1.8	2.2	<3	20°C 30d, 15°C 60d	(Boletzky 1983b)
Sepietta oweniana	Sepioidea	Amorphous mass of spherical capsules	2.7 x 2.3	1	2.5	3-5	5	10.2°C, 60-90d	(Bergstrom and Summers 1983)
Euprymna berryi	Sepioidea	Ovoid capsules	6.3 x 5.2	1			2.6		20d	(Choe 1966)
Sepiella maindroni	Sepioidea	Ovoid capsules	7.5-10.5 x 6.5-8.5	1		4.6		20-26°C 28-30d, 15-24°C 35-53d	(Choe 1966)
Sepia esculenta	Sepioidea	Ovoid capsules	16-21 x 12-14	1		5.7		12-22°C 55-86d, 15-24°C 29-42d	(Choe 1966)
Sepia officinalis	Sepioidea	Flask shaped capsules	25-30 x 12-14	1	6-9 x 5-7	6-9	<50	20°C 40-45 d, 15°C80-90 d	(Boletzky 1983a)
Sepia subaculeata	Sepioidea	Ovoid capsules	31.5 x 14.7	1		8.7		29-31d	(Choe 1966)
Rossia pacifica	Sepioidea	Individual capsules	10	1			4.5	4.5-9 mths	(Anderson and Shimek 1994)
Sepia apama	**Sepioidea**	**Flask shaped capsules**	**20 x 30**	**1**	**10 x 11**	**12**	**20-40**	**12°C 160 d**	**this study**
Illex illecebrosus	Teuthoidea	Jelly mass	1 m spheres	100 000	0.6-0.9 x 0.8-1	1.1	25	13°C 16d	(O'Dor 1983)
Todarodes pacificus	Teuthoidea	Viscous and albuminous secretion	football sized	300-400	0.75-0.83 x 0.73-0.77	0.74-1.02	25-29	14-21°C 4.5 d, 10-13°C 11d	(Okutani 1983)
Dosidicus gigas	Teuthoidea	Gelatinous sausage with egg in outer layer	6-15 x 1-2		0.9-1.1	1	115-120		(Nesis 1983)
Doryteuthis plei	Teuthoidea	Gelatinous strand with eggs in outer coat	20 cm long	180			12.5	10d	(Roper 1965)
Thysanoteuthis rhombus	Teuthoidea	Floating egg mass, with double helix of	100-130 x 15-20 cm		1				(Boletzky 1998)

eggs

Species	Order	Egg mass	Dimensions	Number	Egg size			Temperature/time	Reference
Loligo peallii	Teuthoidea	Mass of finger like capsules	8-10 x 3.5-5 cm	180	1 x 1.6	1.8	30-46	12°C 27d, 23°C 10d	(Summers 1983)
Lolliguncula brevis	Teuthoidea	Gelatinous strand	10-13 cm long	69	2.2 x 1.6	3.8	6.9	35-40d	(Hall 1970)
Loligo opalescens	Teuthoidea	Finger like capsules in large mass 12 x 1.3 m	8 x 1.2 cm	180-300	2-2.5 x 1.3-1.6	2.5-3.2	23	13.6°C 30-35d	(Hixon 1983)
Loligo vulgaris	Teuthoidea	Jelly string	6-16 cm	90	2.3-2.7 x 1.8 x 2.2	2-3	15-17	12°C 40d, 22°C 26d	(Worms 1983)
Sepioteuthis lessoniana	Teuthoidea	Mass of finger like capsules	7.6 x 0.9-1.3 cm	2-9		5.6		25-28d	(Choe 1966)
Sepioteuthis australis	Teuthoidea	Mass of finger like capsules	5-7 cm	4-6			40	20°C 140d	(Zeidler and Norris 1989)
Loligo forbesi	Teuthoidea				3.2 x 1.9	4.5			(Boletzky 1987b)
Gonatus fabricii	Teuthoidea	Mucous sheet containing eggs in single layer	60-90 x 10-21 cm		5.24				(Bjorke *et al.* 1997)

Energetics of embryonic development

Introduction

Two extremes of marine invertebrate reproductive strategy have been defined; the production of planktotrophic eggs with little nutritional content which generally hatch into feeding larvae prior to metamorphosis, and the production of lecithotrophic eggs which generally contain all of the nutritional energy necessary for development to either a pre-metamorphosed non-feeding larvae or a juvenile (Jaeckle 1995). Lecithotrophic eggs spend a greater proportion of development within the egg capsule, and require protection from predators and pathogens. The developmental duration is positively correlated with the energy investment in protective materials (Perron 1981). Therefore, as egg size increases, developmental duration increases and the total energy investment per egg increases. With a finite amount of energy to apportion to reproduction, fewer large eggs are produced. However, larger eggs produce larger hatchlings, which have lower post hatching mortality (Ware 1975, Rosenberg and Haugen 1982, Hutchings 1991).

The lecithotrophic cephalopod egg is essentially a closed system, with the yolk containing all of the energy required for embryonic development. Embryonic development involves the conversion of potential energy stored in the yolk into energy used for growth and maintenance of both the embryo and the micro-environment it develops within. The majority of energy consumed (C) from the yolk (Y) is converted to ATP via respiration and used to fuel metabolic processes (M), including mechanical and chemical work and growth or synthesis, culminating in the production of the embryo (P). Energy conversions are not 100% efficient, resulting in some energy being dissipated as heat (Lehinger 1971,
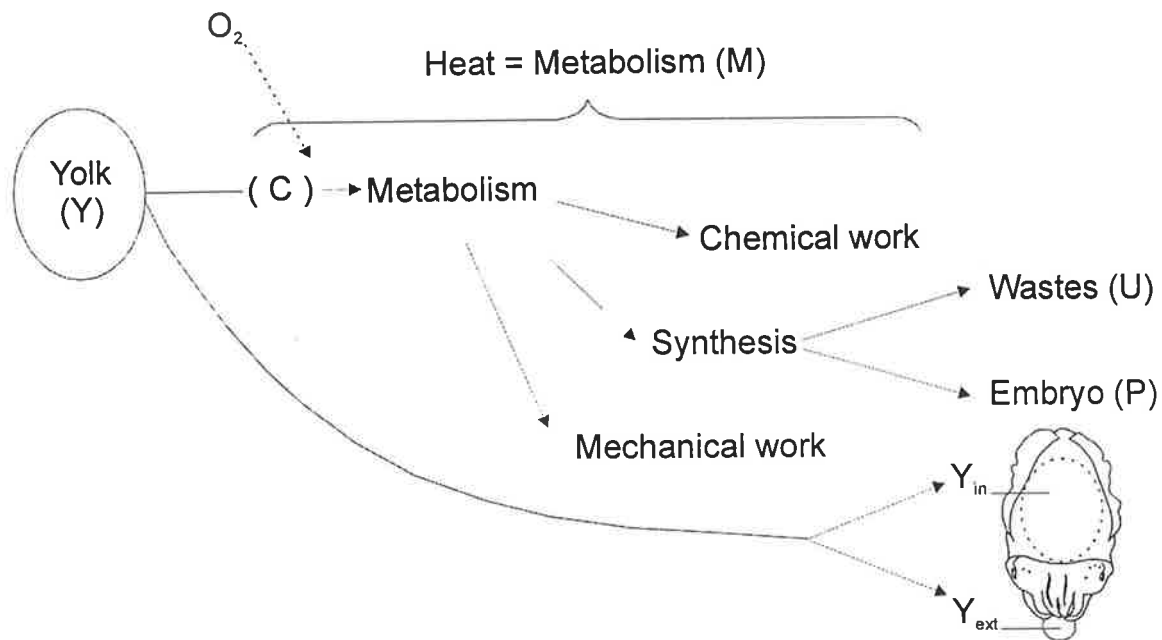
Figure 5.1: Partitioning of energy contained in the yolk. A large portion (C) of the energy contained in the yolk (Y) is metabolised to create energy for processes of maintenance of the egg (M) and growth of the embryo (P), with the concurrent generation of waste products (U). The embryo contains an internal yolk reserve (Y_{in}), and may hatch with a portion of yolk remaining (Y_{ext}).

Brett and Groves 1979) or chemical waste products (U) (Fig. 5.1). The partitioning of energy can be summarised in an energy budget, whereby;

$$C = M + P + U$$

Energy is conserved once assimilated into tissue, but subsequent maintenance of the tissue requires a continual supply of energy to replace degraded molecules, to transport molecules into and within cells, and to provision mechanical work. In addition, energy is required to maintain the micro-environment of the egg, which is fundamental for the development of the embryo. Therefore, only a fraction of the chemical potential energy stored in the yolk is conserved in the embryo. This fraction, (P) of the total energy consumed (C), is a measure of the energy conservation ($100 \times P/C$), historically termed the gross production efficiency

(Calow 1977, Wieser 1994). Another commonly used term is the net production efficiency, which is the amount of energy conserved in the embryo out of the energy assimilated ($100 \times$ P / C-U), where U accounts for losses through waste products (Finn 1994). However, since the portion of energy used to generate P is not included, and only the final energy conserved in P is measured, the ratios are not a measure of efficiency but represent the energy conserved in the embryo (Wieser 1994). The (gross) conservation of yolk in embryonic tissue is approximately 60% in reptile and bird eggs (Needham 1931) and 77% in marine fish (Wieser 1994).

The partitioning of energy can be determined from the caloric or energy content of the yolk (Y), embryo (P) and waste products (U) by determining the heat generated when the material is oxidised in a bomb calorimeter and comparing with the heat generated and energy content of a known standard. However, since cephalopods are ammoniotelic, the collection of waste products is difficult and the amount of waste generated by marine embryos is often considered negligible (Lucas and Crisp 1987, Rombough, 1988a, Wieser 1994). The energy dissipated via metabolism (M) can be measured either by direct calorimetry, which measures the heat generated by the organism, or by respirometry. Respirometry involves measuring embryonic oxygen consumption over the developmental period (Chapter 4) and converting the consumption to energy equivalents. Quantification of the energy associated with metabolism by respirometry assumes that metabolism is fully aerobic. However, anaerobic pathways can constitute a large fraction of the metabolism of bivalve molluscs (Wang and Widdows 1993), and are known to occur in fish embryos (Gnaiger *et al.* 1981, Cetta and Capuzzo 1982, Vetter and Hodson 1983). If anaerobic pathways contribute significantly to metabolism the conversion will underestimate the energy associated with metabolism. If metabolism is solely aerobic, the rate of oxygen consumption should be related to the metabolic rate determined from the difference between the energy consumed (C) and the energy contained in the hatchling (P), provided energy dissipated through wastes (U) is negligible.

The energy budget of embryonic development was measured for *Sepia apama* to determine the energy conserved in the hatchling and to assess whether anaerobic pathways contribute to metabolism. The relative energy content of the capsule and yolk was measured and implications for the reproductive strategy discussed.

Methods

Initial egg energy content

Each season a subset of young eggs (with no embryo observable and no PVF) were selected representing a range of egg sizes. Eggs were blotted with absorbent paper and weighed. Eggs were frozen in liquid nitrogen for 45 s and dissected to obtain the yolk, capsule and jelly components of the egg. These were weighed individually and then frozen at -4°C in preparation for freeze-drying.

Final energy content

As near as practicable to hatching or upon hatching, the hatchling (H) plus any remaining residual (external) yolk (Y_{ext}) was collected. The Y_{ext} was carefully disconnected from the mouthparts of the hatchling and weighed separately. Hatchlings were transferred from the aquaria via absorbent paper to a tared container of water on the balance. Hatchlings were fixed with 10% gluteraldehyde to enable easier dissection of the internal yolk (Y_{in}) from the body cavity. The mass of the hatchling without internal yolk (H_{yf}) and the mass of the Y_{in} were measured. Hatchlings, Y_{ext} and Y_{in} were placed in 5 ml plastic containers and frozen at -4°C in preparation for freeze-drying and dry mass determination.

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Energy content

Frozen samples were freeze dried in a Dynavac FD-5 freeze drier for 24 h. The masses of the dried samples were measured on a balance. Samples were ground with a mortar and pestle and compressed into cylindrical pellets approximating 0.2 × 0.5 mm with a mass ranging from 0.02 - 0.06 g. Pellets were stored in a desiccator filled with silica gel granules.

The energy contents of egg constituents were measured using a Parr 1261 Isoperibol Bomb Calorimeter with a 1107 Semi-micro Oxygen Bomb. A bomb calorimeter measures the heat increment when a substance is oxidised. The heat generated and the oxygen consumed are related stoichiometrically, thereby the amount of energy originally contained in the substance can be quantified by determining the heat of combustion of a standard substance. The calorimeter was standardised with benzoic acid tablets (heat of combustion = 26.456 kJ per gram @ 25°C). At least 10 calibration runs were performed to obtain a heat of combustion standard with < 2% standard deviation. Pellets of the egg constituents were weighed and placed in the microbomb. This was sealed and filled with oxygen to 35 atm. The calorimeter determined the heat of combustion from the initial pellet weight and the difference in temperature between the bucket and jacket following firing. Any residual material remaining in the microbomb was weighed to determine the ash free dry mass (afdm) of the combustible pellet.

Results

Initial egg energy content

Combustion of yolk yielded an energy density of 22.19 ± 0.21 (25) kJ g_{afdm}^{-1} for yolk, and less than half that for the capsule material (10.41 ± 0.25 (24) kJ g_{afdm}^{-1}). Combustion of the yolk was complete, whereas only 65% of the capsule material was combusted, leaving a resin like residue, whilst the jelly material did not combust. The yolk energy density was

independent of egg size, however the greater yolk mass of larger eggs (Chapter 2) resulted in larger eggs having a greater energy content (Fig. 5.2). Similarly, larger eggs had proportionately more capsule material (Chapter 2) resulting in a greater energy content (Fig. 5.3). The total egg energy content ranged from 2.75 to 6.32 kJ over the egg mass range of 0.22 to 0.59 g and averaged 4.34 ± 0.2 (29) kJ for an egg of mass 0.38 ± 0.02 (40) g (Fig. 5.4).

The initial average mass, energy density and energy content of the yolk from eggs representative of those used for determination of oxygen consumption (Chapter 4) are presented in Table 5.1.
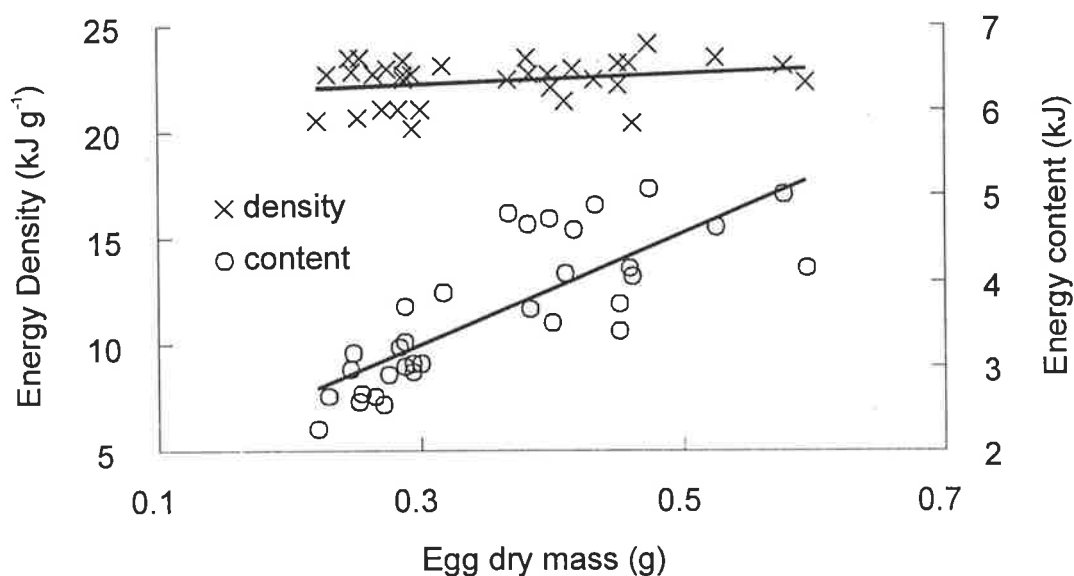
Figure 5.2: The effect of initial egg dry mass on energy density and energy content of yolk. The linear equations describing the data are: yolk energy density = 2.399 × egg dry mass + 21.526, r^2 = 0.58; yolk energy content = 6.474 × egg dry mass + 1.312, r^2 = 0.64.
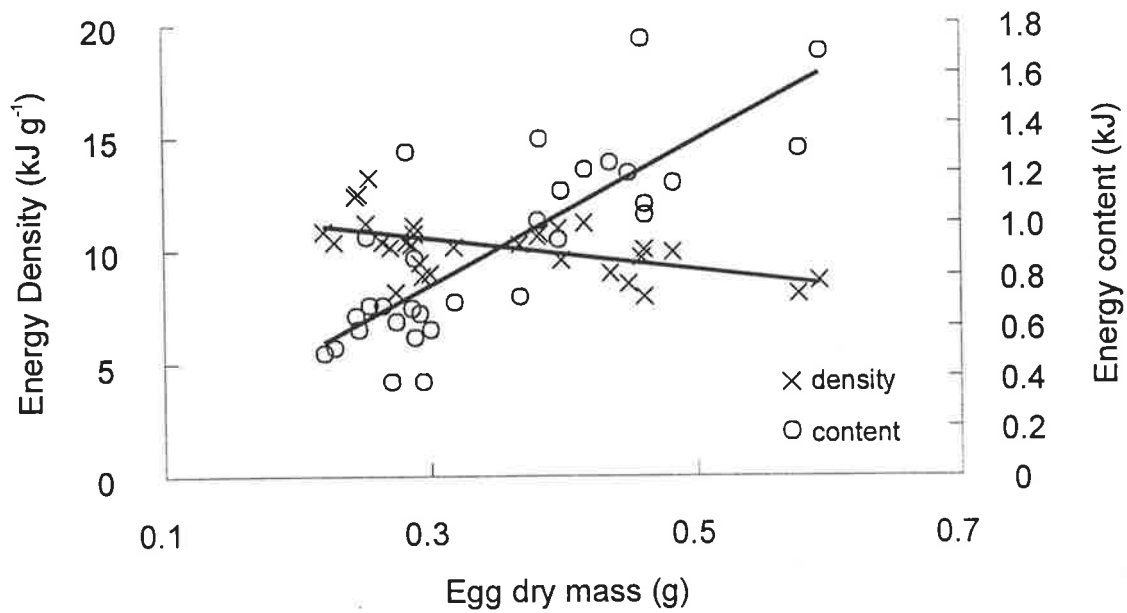
Figure 5.3: The effect of initial egg dry mass on the energy density and energy content of the capsule. The linear equations describing the data are: capsule energy density = -6.841 × egg dry mass + 12.506, r^2 = 0.30; capsule energy content = 2.836 × egg dry mass - 0.089, r^2 = 0.64.
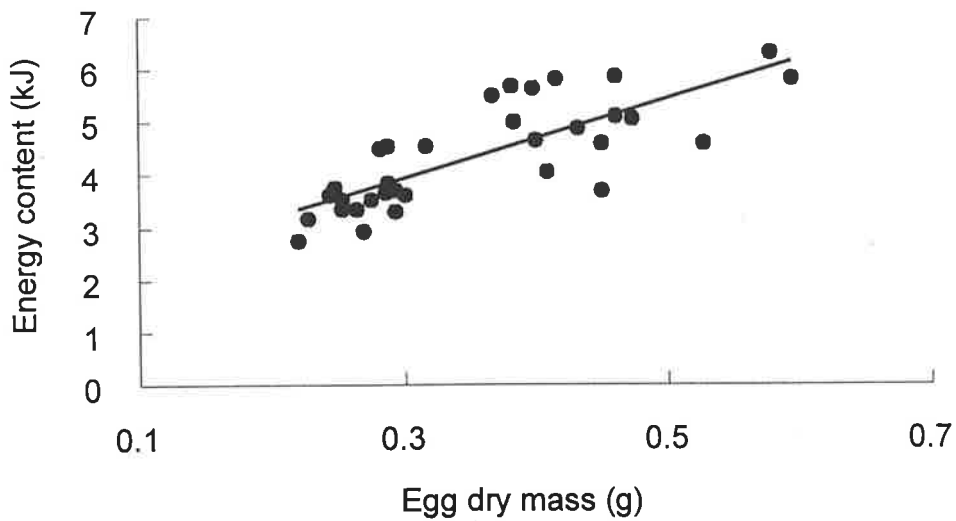
Figure 5.4: Total energy content of different sized eggs. The linear equation describing the data is: egg energy content = 7.503 × egg dry mass + 1.689, r^2 = 0.62.

Final composition

At the completion of development, there is no jelly present, and the egg comprises the embryo with internal yolk, any remaining external yolk, the PVF and capsule. The mass, energy density and energy content of the embryo and yolk are presented in Table 5.1. In the process of dissecting the Y_{in} from the embryo, some yolk material was lost, resulting in the Y_{in} mass being underestimated. Therefore, the Y_{in} mass was determined from the difference between the hatchling (H) mass and the dissected hatchling (H_{yf}) mass. From a total of 38 hatchlings, 18 were collected that retained their Y_{ext}. External yolk mass ranged from 3.50 to 6.64 mg. The heat of combustion of the Y_{ext} was not measured, but was estimated as 21.22 kJ g^{-1} from the average of the Y_{in} and initial yolk energy density.

The initial energy available is the energy contained within the yolk (Y = 3.39 kJ), of which only a portion is used during incubation. The energy used (C) is the initial energy (Y), less the energy content remaining from the residual (Y_{ext}) and internal (Y_{in}) yolk (C = 3.39-0.57-1.14 = 1.68 kJ). The production of the embryo culminates in energy conserved in the hatchling (P = 0.97 kJ). Assuming that waste production is negligible (U = 0) the energy dissipated via metabolism is the difference between the energy consumed (C), and the energy conserved in the hatchling (P);

M = C - P = 1.68 - 0.97 = 0.71 kJ

Alternatively, by using the energy content of the non-dissected hatchling to minimise errors, M = initial energy content (C) - external yolk energy content (Y_{ext}) - non-dissected embryo energy content (H);

M = 3.39 - 0.57 - 1.76 = 1.05 kJ

Therefore, C = 1.05 + 0.97 = 2.02

Thus the production of 52 mg of dry *S. apama* hatchling containing 0.97 kJ requires 2.02 kJ of yolk energy, 1.05 kJ of which is used to fuel metabolic processes. Therefore the conservation of yolk into embryonic tissue (P/C) is $0.97/2.02 \times 100 = 48$ %.

Table 5.1: Composition and energy content of the yolk and of the hatchlings of *Sepia apama*. Initial yolk (Y), hatchling with internal yolk (H), hatchling without internal yolk (H_{yf}), internal yolk (Y_{in}), residual yolk (Y_{ext}). Data is presented as mean ± SE (n). *see Results: Final composition.

	wet mass (mg)	dry mass (mg)	% water	energy density (kJ $g^{-1}_{(afdm)}$)	ash (%)	energy content$_{(afdm)}$ (kJ)
Y	363.2 ± 17.5 (25)	152.2 ± 6.5 (25)	57.4 ± 1.1 (25)	22.2 ± 0.2 (25)	-	3.39 ± 0.15 (25)
H	496.2 ± 9.5 (20)	108.3 ± 5.8 (20)	82.5 ± 3.0 (13)	18.2 ± 0.3 (12)	12.0 ± 1.3 (12)	1.76 ± 0.12 (12)
H_{yf}	338.5 ± 34.9 (17)	57.5 ± 5.6 (17)	84.7 ± 0.7 (17)	17.9 ± 0.7 (16)	13.5 ± 5.4 (15)	0.97 ± 0.15 (16)
Y_{in}	207.3 ± 14.6 (7)	56.3*	-	20.3 ± 0.7 (7)	-	1.14*
Y_{ext}	53.6 ± 8.6 (18)	26.8 ± 4.4 (18)	47.5 ± 2.7 (18)	21.2*	-	0.57*

Discussion

The determination of energy dissipated by metabolism (M = 1.05 kJ) by the difference between yolk used (C) and conserved in the embryo (P) compares with the energy of metabolism determined by respirometry of 0.26 kJ for a 160 day developmental period at 12°C (assuming 1 l O_2 yields 20.1 kJ, and embryonic development requires 13.14 ml O_2;

Chapter 4). Thus a discrepancy of 1.05-0.26 = 0.79 kJ results from the different methods (bomb calorimetry versus respirometry) of analysing the metabolic rate, indicating some processes have not been taken into account in the equations. A similar comparison of methods provided balanced energy budgets in cod, halibut and turbot (Finn 1994), however huge discrepancies were obtained with dogfish, most probably as a result of excluding energy associated with waste production and anaerobic metabolism (Diez and Davenport 1990). The possible sources of the discrepancy were investigated for *S. apama*.

The energy density of *S. apama* yolks of 22.2 kJ g^{-1} determined from bomb-calorimetry is consistent with the oxidation equivalent of 24.7 kJ g^{-1} of mixed lipid-protein (Brett and Groves 1979), but greater than the energy densities of 17.9 kJ g^{-1} of the yolk of *Sepia officinalis* eggs (Bouchaud 1991). The energy density of the yolk of *S. apama* calculates to 9.3 J mg^{-1}(wet) which is also greater than the energy density of 7.15 J mg^{-1}(wet) determined from the ripe ovaries of *Loligo opalescens* (Giese 1969) (Table 5.2). Conversely, the average energy density of the yolk of gastropods was 26 kJ g^{-1}(dry) (Perron 1981), and of barnacles was 28 kJ g^{-1}(dry) (Lucas and Crisp 1987). Thus the energy density of *S. apama* eggs appears reasonable.

The principal energy stores of carnivorous fish are comprised of lipid and protein (Brett and Groves 1979), which yields 24.7 kJ g^{-1}. If the *S. apama* hatchlings are assumed to comprise of mixed lipid-protein, the energy density of 17.9 kJ g^{-1} determined from bomb calorimetry is lower than expected. The energy density of the wet *S. apama* hatchling calculates to 2.86 kJ g^{-1}. A comparison with literature sources (Table 5.2) suggests this value is slightly lower than other determinations of energy density of cephalopod tissue. The possible underestimation of *S. apama* hatchling energy density, would undervalue the conservation of energy in tissue (P), and overestimate the energy associated with metabolism (M). The metabolic cost (M/C) approximates 52% of the total energy used, which is greater in comparison to other studies (Brett and Groves 1979, Finn 1994), and results in a calculation

of 48% for the energy conserved in the embryo, which is much lower in comparison with *S. officinalis* (Table 5.3) and with other literature sources for embryonic development. The conservation of yolk energy in embryonic tissue approximates 55% in fish embryos (Finn 1994), is respectively 62, 65, 51 and 59 % in the chick, silk-worm, frog and sea-urchin embryo (Needham 1931), 62-67% in freshwater gastropod embryos (Calow 1977) and 69% in marine invertebrate larvae (Holland 1978). If a value of 3.5 kJ $g^{-1}_{(wet)}$ is adopted for the energy density of *S. apama* (averaged from Table 5.2), the value of P increases to 1.24 kJ, and the energetic efficiency to 61%, consistent with the literature. Furthermore, the energy associated with metabolism (M) of the yolk to produce the hatchling is reduced to 0.78 kJ or 39 %, comparable to the values determined for juvenile and adult species of carnivorous fish (Brett and Groves 1979). This reduces the difference between the two methods of determination of the metabolic rate to 0.78-0.26 = 0.52 kJ, which still exceeds the value for metabolic energy expenditure calculated by respirometry. Other possible sources for error include the loss of energy associated with waste production, the contribution by anaerobic metabolism, and the use of yolk energy by capsule bacteria.

Table 5.2: Energy density of yolk and body tissue of different species of cephalopod.

Family or Species	Comments	Energy density $(J\ mg^{-1}_{\ wet})$	Source
Sepia officinalis	eggs and hatchlings	18$_{(dry)}$	(Bouchaud 1991)
Loligo opalescens	ripe ovaries	7.15	(Giese 1969)
Ommastrephidae,	7 species, whole	3.01-4.19	(Croxall and Prince
Loliginidae, Sepiidae	animal		1982)
Nototodarus sp.,		4.36	(Vlieg 1984)
Sepioteuthis australis,		3.38	
Moroteuthis ingens		3.5	
Squid	11 species, whole animal	1.69-4.53 20.0$_{(dry)}$	(Clarke *et al.* 1985)

Assuming ammonia yields 20.5 kJ g^{-1} (Withers 1992), 25.4 mg of ammonia would have to be produced during development to compensate for the difference between the two methods of determining metabolism. The difference represents 26% (0.52/2.02) of the total energy used during development. Estimates of waste production in marine organisms are limited, however Finn (1994) calculated that 6% of the total energy available from the yolk was released as ammonia waste in atlantic cod larvae, whilst nitrogenous wastes accounted for 1-2.5% of the energy mobilised before exogenous feeding in trout (Smith 1947, Rice and Stokes 1972). Therefore, the estimate of waste production in *S. apama* is too large alone to account for the energy difference between the methods of determining total metabolism, but would contribute significantly to the energy dissipated.

If metabolism of the energy source were not solely aerobic, the metabolic rate determined by respirometry would underestimate the energy converted. Anaerobic glycolysis utilises glucose as a substrate to produce lactic acid and ATP via the production of many intermediates including glyceric and pyruvic acid. In many invertebrates, alternative anaerobic pathways may be utilised which produce various end-products analogous to lactate (Hochachka and Somero 1984). In the cephalopods a likely end-product of anaerobic metabolism is octopine (Storey and Storey 1983). Therefore the presence of glycolytic products implies anaerobic metabolism. Anaerobic by-products occur in marine fish embryos (Boulekbache 1981, Gnaiger *et al.* 1981, Cetta and Capuzzo 1982, Vetter and Hodson 1983) and anaerobic pathways can constitute a large fraction the metabolism of bivalve molluscs (Famme *et al.* 1981, Wang and Widdows 1993). Wolf *et al.* (1985) reported on the presence of anaerobic-end products and embryonic haemocyanins (pre-Hcns) in *S. officinalis* embryos, which are presumed to assist in meeting the energy requirements of the embryo. The protein fraction of the blood of embryos changes throughout development suggesting the blood haemocyanins differ over time, possibly correlating to the oxygen requirements of the embryo and the P_{O_2out} (Decleir and Richard 1970, Decleir *et al.* 1971).

Table 5.3: Energy budgets for developing embryos of *Sepia apama* (this study) and *Sepia officinalis* (Bouchaud 1991). * Energy budget calculated assuming *S. apama* energy density of 3.5 kJ $g^{-1}_{(wet)}$, see Discussion.

Species	Y	(Y_{ext})	(Y_{in})	(C)	metabolism (M)	embryo (P)	excretion (U)	Energy conservation (100 × P/C %)	metabolic dissipation (M/C %)
Sepia apama	3.39	0.57	1.14	2.02	1.05	0.97	-	48	52
*(assuming 3.5 kJ g^{-1})					0.78	1.24		61	39
Sepia officinalis	1.392	0.354	0.335	0.703	0.136	0.567	-	81	19

Anaerobic glycolysis utilises a large amount of substrates and yields far less energy (4.71 mol ATP per mol of glycogen) than aerobic metabolism (37 mol ATP per mol of glycogen) (Wang and Widdows 1993). However, anaerobic glycolysis in adult *Mytilus edulis* exposed to an environmental PO_2 of 10 kPa contributed to as much as 40% of the total metabolic rate (Famme *et al.* 1981), whereas (Wang and Widdows 1993) found the contribution by anaerobic metabolism to be negligible in mussels of the same species under similar conditions. The responses of organisms to environmental PO_2 influences the contribution by anaerobic glycolysis to total metabolism (Chapter 4). *S. apama* embryos are subjected to low PVF PO_2 during development (Chapter 4), which is likely to initiate anaerobic metabolism. Certainly the presence of anaerobic end-products in *S. officinalis* embryos suggests anaerobic pathways are occurring, and may be sufficient to account for, or at least contribute to, the energy difference determined for the two methods of measuring metabolism in *S. apama* embryos.

Some marine invertebrate embryos obtain nutrients from the PVF (Rivest 1986, Garin *et al.* 1996, Miloslavich 1996) during development, however, the low energy density of *S. apama* capsules suggests they are purely protective in function. In the absence of capsule nutrition, the source of energy for bacteria occupying the capsule may originate from the yolk. Bacterial consumption of yolk would represent an energy sink unaccounted for in the energy budget. The potential amount of energy consumed by respiration of the bacteria accounts for 0.14 kJ, which is approximately half of the oxygen consumed by the embryo throughout development, but only 27% (0.14/0.52 = 0.27) of the energy difference remaining from the two methods of determining M. However, it is also likely that the bacteria obtain nutrients from dissolved organic matter (DOM) in the seawater, which contributes significantly to the embryonic nutrition of many marine invertebrate eggs (Monroy and Tolis 1961, Epel 1972, Manahan and Crisp 1982, Manahan 1983) and larvae (Fontaine and Chia 1968, Reish and

Stephens 1969, Manahan and Crisp 1982, Manahan 1989, Jaeckle and Manahan 1989), in which case the yolk energy potentially consumed by the bacteria would be overestimated.

In summary, the difference in energy dissipated between the two techniques of measuring metabolism may be accounted for by the summation of energy associated with the production of wastes, by anaerobic glycolysis, and possibly by consumption by capsule bacteria.

Ecological implications

Field observations made over a three year period from two locations have shown that the eggs of *S. apama* have a large size variation (Chapter 2) and energy content, which averages 4.34 kJ per egg produced, apportioned between the ovum (80%) and capsule (20%). Variation in egg size is not uncommon within a species and may reflect numerous factors including maternal size (Salthe and Mecham 1974, Chaparro *et al.* 1999) and salinity (Blaxter 1969). The initial egg size and the efficiency of conversion of yolk determines the size of the hatchling. Larger *S. apama* eggs produced larger hatchlings (Chapter 2), and large hatchling size correlates positively to survival rates in fish (Blaxter 1969, Hutchings 1991) and gastropods (Spight 1976). Since the energy contained in the yolk represents all of the nutrient sources for the embryo (excluding eggs where DOM absorption contributes a significant proportion of the energy available to the embryo), an egg provisioned with a greater amount of resources may result in an offspring having a greater chance of success. The large ovum of *S. apama* eggs not only provides energy for embryonic development and egg maintenance, but also provisions the hatchling with a large store of energy contained in the internal yolk. The internal yolk enables a non-feeding period during which the juvenile can acclimatise to the environment and develop feeding techniques. This energy store represents 50% of the dry mass of the *S. apama* hatchling (Chapter 2), and between 38-50% in *S. officinalis* depending on the temperature (Table 5.3). In *S. apama* the internal yolk constitutes 1.14 kJ of energy or 58.81 ml of O_2 (assuming lipid/protein combustion yields

19.38 kJ l^{-1} O_2). Therefore, the internal yolk could provision a hatchling that has a $\dot{V}O_2$ of 13.6 μl h^{-1} (metabolic rate @ 12°C: Chapter 4) for 180 d. Clearly the $\dot{V}O_2$ of an active, growing juvenile at higher ambient temperatures (18°C) would be greater than that of a resting hatchling, increasing the rate of yolk consumption. If a $\dot{V}O_2$ of 30 μl h^{-1} is assumed, the yolk would provide energy for approximately 80 days. The time for yolk absorption varies from several hours (*Hapalochlaena maculosa*) to a couple of months (*Rossia macrosoma*) depending on the environmental conditions (Vecchione 1987). The development of the adult digestive glands following the first feeding event may take 1-2 months in *S. officinalis* (Boucher-Rodoni *et al.* 1987). Therefore the estimate of 80 days offers a reasonable energy reserve to make the transition from yolk absorption to feeding. The production of hatchlings with a large energy store would be favoured in conditions where food was limited, as it maximises the survival duration.

The total energy investment per egg includes the energy associated with the production of capsule materials. The energy density of the capsule of *S. apama* at 10 kJ g^{-1} was much lower than the energy density of capsules of *Conus* species which averaged 22.8 kJ g^{-1} (Perron 1981). The higher residual or ash content of *S. apama* capsules and lower energy density suggests the capsule is purely protective in function and does not provide nutrition for the embryo as in some gastropod species (Miloslavich 1996). The investment in protective materials correlates positively with the developmental duration among different sized gastropod eggs, as larger eggs need to be protected for longer (Perron 1981). In *S. apama* a greater energy investment in protective materials (ie: thicker capsules) is expected to provide greater protection for the embryo (Chapter 2). As egg size increases in *S. apama* the mass (Chapter 2) and energy content of capsule material increases disproportionately (Fig. 5.3), and therefore total energy investment per egg increases disproportionately (Fig 5.4).

With a finite amount of energy to allocate to reproduction, energy spent on the production of protective materials increases the production cost per egg (Perron, 1981), (Lee and Strathmann 1998), reducing the energy invested in ova which results in fewer eggs. Selection should therefore favour egg sizes with the minimal amount of the energy invested in capsule material, but which maximises protection throughout the development and maintains large ovum size if the success of the juvenile depends on the hatchling size. Alternatively, modifications of egg capsule design may occur, which minimise the protective investment per egg.

In cephalopods, the evolutionary trend is from the production of single eggs with simple multi-layered coats directly attached to the substrate, to the production of a complex multi-egg capsule cemented to a substrate, or entwined into the centre of a common egg mass (Arnold and Williams-Arnold 1977). The latter arrangement reduces the investment in protective material per egg and so reduces total cost per egg, thereby maximising the energy allocated to the production of ova. However, the incorporation of eggs into masses has implications for gas exchange, with asynchronous development or mortality occurring if oxygen supply is insufficient (Chapter 4), therefore large eggs incorporated into masses require more gelatinous material to space the eggs to enable gas exchange, which again increases the total energy investment per egg (Lee and Strathmann 1998). Eggs of incirrate octopods minimise the energy allocation to the production of jelly for attachment purposes only as the egg is surrounded solely by the chorion, which potential enables a greater investment in ovum production. However, energy would have to be 'reserved' in the adult for brooding and protecting the eggs after laying, possibly resulting in a total reproductive investment equalling or exceeding that of species producing non-brooded eggs.

To summarise, the reproductive energy of *S. apama* is distributed between the production of ova (80%) and gelatinous protective material (20%). The protective investment per egg is expected to increase with the developmental duration (hence egg size), which will reduce the

total number of eggs produced. The situation in further complicated if post-spawning egg care occurs, which detracts energy from egg production. Added to this are the physical constraints imposed by gas exchange, which limit the thickness of the egg capsule or mass (Chapter 4). Numerous factors contribute to the ultimate design of eggs and egg masses, resulting in a diversity of egg designs which satisfactorily facilitate the environmental conditions experienced.

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