



# **Development in the Port Jackson shark embryo**

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## ABSTRACT

The Port Jackson shark (*Heterodontus portusjacksoni*) is an oviparous shark that breeds annually, between the months September and November. Temperatures in the field vary between 12°C to 22°C, producing long incubation periods of over 12 months.

Incubation period in the laboratory is strongly temperature dependent, with embryos hatching after 400 days at 18°C and after 310 days at 22°C.

Each egg contains one embryo and is encased in a thick (1-2mm) pear-shaped collagenous casing that protects it. A freshly laid egg weighs  $170.2 \pm 5.0$  g and contains a large yolk ( $38.6 \pm 1.3$  g), surrounded by a thick gelatinous albumen ( $70.7 \pm 5.0$  g).

The capsule is  $128.6 \pm 2.4$  mm long and weighs  $60.9 \pm 3.2$  g at laying. The egg capsule is equipped with two respiratory slits, one located at either end of the case. From the time of laying up to the fourth month of incubation, the respiratory slits are plugged with a thick wedge of albumen, and the egg capsule is referred to as sealed. At four months, the plugs dissolve and the embryo is in direct association with the external seawater environment. The egg capsule is referred to as open.

Fifteen morphological stages are described for the developing embryo. In order to increase its effective surface area and therefore its aerobic capacity during the first four months of incubation, the embryo develops vascular external gill filaments, fins and yolk surfaces. The external gill filaments are extensions of the internal gill vasculature. They are thin walled and protrude from the gill openings into the albumen. When the egg capsule is open, the external gill filaments are reabsorbed back into the internal gill structure and the embryo uses its internal gills for respiration.

The embryo grows exponentially until it reaches a short plateau phase near hatching. By hatching it has transformed 77.7, 82.1 or 85.3% at 18°C, 20°C or 22°C, respectively, of the initial dry mass of yolk into hatchling tissue mass. From stage 12 onwards, the nutrients from the external yolk are simultaneously utilised for development and directed into an internal yolk sac which is attached directly to the spiral gut. By the time the embryo hatches as a self-sufficient fish, it has depleted all external yolk and virtually all internal yolk stores.

The initial yolk provides an average of 541.9 kJ of energy for the growth and maintenance of the embryo. The efficiency of growth, measured as the ratio of energy content of the hatchling to the energy content of the yolk, is high compared to embryos of other species (78.9, 83.4 and 86.7% at 18°C, 20°C or 22°C, respectively). The efficiencies are statistically similar at all temperatures despite the significant increase in incubation period at the lower temperatures, although a trend for reduced efficiencies at lower temperature was found.

Between egg capsule opening and hatching, oxygen consumption is correlated to the wet mass of the embryo plus yolk by the equation  $\dot{V}O_2 = aM^b$  where  $a = 53.6, 77.1, 173.4$  and  $557.2$  and  $b = 0.83, 0.77, 0.52$  and  $0.22$  at 15, 18, 20 and 22°C, respectively. As the embryo increases in mass,  $Q_{10}$  values for  $O_2$  consumption decrease from 7.9 at 5 g to 0.9 at 55 g near hatching. Stage 12 embryos maintain a steady  $O_2$  consumption rate of  $0.1 \mu\text{mol } O_2 \text{ min.g}^{-1}$  at a  $PO_2$  between 69 and 150 Torr. Below 69 Torr,  $O_2$  consumption falls linearly with ambient  $PO_2$ . The total  $O_2$  consumed by the embryo increases as the incubation is lengthened at the lower temperatures. An embryo consumes 417, 285 and 267 mmol of  $O_2$  at 18, 20 and 22°C, respectively. Hatchlings at all temperatures are of similar wet (54.9-58.8 g) and dry masses (16.1-17.7 g) and the cost of development is 25.9, 16.4 and 15.1 mmol  $O_2$  per g dry hatchling at 18°C, 20°C and 22°C.

The embryo actively ventilates the open egg capsule with vigorous scooping movements of the tail that draws water into and out of the egg capsule through the respiratory slits. The last 8 segments of the tail are curved upwards to form a spoon-shaped ending. Stage 10-12 embryos direct the flow of seawater in one side and out of the other side of the large respiratory slit located at the top of the egg capsule simultaneously, ensuring a fresh supply of oxygen. Stage 13-15 embryos are limited in the space available and simultaneously direct water in through the small respiratory slit at the bottom of the case, and out through the larger slit. A mean ventilation rate of  $791 \text{ ml } O_2 \text{ min}^{-1} \cdot \text{kg}^{-1}$  is sufficient to sustain aerobic metabolism in the stage 12 embryo.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text.

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## Chapter 1 INTRODUCTION

Chondrichthyan fishes (elasmobranchs) represent the oldest surviving group of jawed vertebrates, with fossil evidence dating back approximately 450 million years. They have remained relatively unchanged for the last 100 million years (Stevens 1987), reflecting a highly successful evolutionary group. They have a cosmopolitan distribution and occupy a wide variety of habitats, yet there is surprisingly little known about them.

The extant elasmobranchs can be divided into 8 orders, 31 families and approximately 350 species (Compagno 1984). In an evolutionary sense, elasmobranchs are considered to be primitive, based on their conservative external features. However this group of fishes exhibit several features which would suggest otherwise. Within these fish the appearance of many so called "advanced" vertebrate features can first be observed (Amoroso 1960, Wourms 1977, Hamlett 1989a, Wourms and Demski 1993). Such characteristics include internal fertilisation and in some, sperm retention at specialised sites, live bearing (viviparity), the presence of a placenta or a similar structure linking embryo to mother, genital tract development and sex differentiation similar to amniotes (the presence of comparable female and male organs). The reproductive endocrinology is comparable to higher vertebrates. In addition, they have large brains and well developed nervous systems.

Research in the last two decades has been directed towards utilising elasmobranchs in biological and biomedical models, based on the appearance of these advanced features. Placental sharks are suitable objects of study for comparative vertebrate reproductive biology because of the similarity to mammals in endocrine regulation (Hamlett 1990). For example, the regulation of spermatogenesis has been investigated in dogsharks to demonstrate the stage related biochemical changes in Sertoli cells (Dubois and Callard 1990). There is also considerable interest in investigating the immune systems of elasmobranchs, owing to the rarity of cancer in this group. Biochemical studies on the properties of the cartilage are being conducted to develop cancer cures in humans (Hamlett 1988).

Among the elasmobranchs there are two reproductive patterns: oviparity and viviparity, the former being the primitive condition (Price and Daiber 1967, Bone and Marshall 1982, Hamlett 1989b, Wourms and Demski 1993). Both reproductive patterns involve a

large maternal investment for the production and survival of relatively few young compared to the teleost reproductive pattern of producing thousands of eggs, of which only a few will survive.

Oviparity is the reproductive pattern in which the fertilised egg is contained within a protective outer capsule and expelled from the female's body. The young develop externally from the female in the absence of maternal care or maternal-embryonic connections. The embryo is provided with all its organic requirements through a large yolk mass. Inorganic material and water are derived from the external environment (Amoroso 1960).

Egg capsules of oviparous chondrichthyans have been identified and described for a long time (Balfour 1878, Stead 1907, Clark 1926, Smith 1942). Studies on the construction and permeability of egg capsules have been conducted by many investigators (Krishnan 1959, Price and Daiber 1967, Knight and Hunt 1974, 1976, Rusaouen et al. 1976, Hornsey 1978, Foulley and Mellinger 1980a,b, Foulley et al. 1981, Mellinger 1983, Ellis and Shackley 1995, Knight et al. 1996, Knupp and Squire 1998). The egg capsules of elasmobranchs are primarily constructed of cross layers of collagen fibres (Knight and Hunt 1974, 1976, Rusaouen et al. 1976, Wourms 1977, Ellis and Shackley 1995, Knight et al. 1996, Knupp and Squire 1998) producing a tough protective cover for the developing embryo. The evidence for permeability of the egg capsule to large osmotically active substances such as urea is conflicting. Several studies found that the capsules are permeable to urea (Foulley and Mellinger 1980a, Evans 1981, Foulley et al. 1981, Kormanik 1993), while others report that the capsules are impermeable to urea (Smith 1936, Price and Daiber 1967). This has important consequences for early embryonic osmo-regulation that will be discussed in Chapter 2.

Viviparity involves the development of the embryo in utero where it is protected and provided with nutrients throughout gestation (Wourms 1977). This offers a more protective environment for the embryo than oviparous development and several nutritional pathways are available (Hamlett 1989c). Embryos may be lecithotrophic - dependent upon an external yolk sac for the entire gestation period (*Squatina californica* - Natanson and Cailliet 1986, *S. squatina* and *S. oculata* - Capape et al. 1990, *Chlamydoselachus anguineus* - Tanaka et al. 1990) or for just the initial period of development. In the latter case, the embryo exhausts the yolk supply and thereafter

actively searches for intra-uterine food supplies such as siblings (Sand Tiger shark, *Odontaspis taurus* - Gilmore et al. 1983) or infertile eggs which the female continues to produce throughout the remaining gestation period (Pseudocarchariid Shark, *Pseudocarcharias kamoharai* - Fujita 1981). Alternatively, after an initial period of yolk dependence, the embryo may develop a maternal-embryonic placental connection (Sandbar Shark, *Carcharinus plumbeus* - Baranes and Wendling 1981). Placental nutrition is less common (Hamlett and Wourms 1984) but nevertheless first appeared among the elasmobranchs (Hamlett 1989b).

Viviparity enhances the survival of the young because predators are absent in the uterine environment, with the exception of intra-uterine cannibalism. However, in comparison to external development, the female incurs additional energetic costs involved with internal gestation. These include the possibility of reduced agility near the end of gestation because of the female's increased size. Viviparous embryos are generally a substantial size at birth (one meter long in *O. taurus* - Gilmore et al. 1983), and this leads to fecundity restrictions based on the female's ability to carry a large number of embryos. The continual production of eggs as a nutritive source, as occurs in some species, must incur additional energetic costs to the female.

In comparison, oviparity allows the female to produce a greater number of offspring (Bone and Marshall 1982), although the prospects for survival are less outside the female's body, because predators can gain access to the eggs. However, the survival can be enhanced by placing of the egg capsule in a secure location, aided by external features of the capsule itself (for example long tendrils or ridged flanges) which anchor the egg capsule firmly. If the egg capsule is not secured, currents can carry the capsule away or wash it up onto the beach where survival is unlikely. Once the capsule is laid, the female does not invest any additional energy into the embryos' development.

Oviparity has been demonstrated in three families of sharks (Heterodontidae, Orectolobidae, Scyliorhinidae), the chimaeras and skates (Wourms 1977, Bone and Marshall 1982). The subject of this study is the Port Jackson shark, *Heterodontus portusjacksoni* embryo. This species is distributed throughout the southern half of Australian waters along the coast from Queensland to Western Australia. It is a nocturnal species showing a bottom dwelling habit. The migration patterns and breeding habits of *H. portusjacksoni* has been studied over a period of nine years by

McLaughlin and O'Gower (1971), although the embryonic life histories were not examined in any detail by these authors. The advantage of choosing *H. portusjacksoni* is that it is a common species with an abundant supply of eggs. Eggs are laid within a 2-3 month period on an annual basis, with incubation taking approximately one year. This has the additional advantage of being able to collect any one stage of development at particularly times of the year.

Oviparity offers an excellent opportunity to study the developing embryo and its interactions with the environment directly. The ability to invade the embryos' environment without lethal consequence allows the monitoring of parameters such as morphological development, growth, energetics, respiration and ventilation throughout incubation. The variation produced by using many individuals to describe the ontogeny of development is reduced by the ability to follow one individual through the entire incubation. This is particularly important where there is a tremendous variation in egg size (such as in *H. portusjacksoni* - 90-210g) which can subsequently be observed in growth, energetics and metabolism. Studies of oviparous elasmobranch embryos have great advantages over the study of avian and reptilian eggs where the process of opening an egg is usually fatal and a sequential series of data cannot be taken.

In general, the embryology of elasmobranchs has been largely overlooked. My study is the first to link morphological and physiological changes that accompany development. Development is the collective result of many parameters and to understand it fully, you need to look at the whole picture. Prior to this study, only one or two aspects of development have been investigated in any one study (Te Winkle 1950, 1963a,b, Diez and Davenport 1987, 1990, Pelster and Bemis 1991, 1992, Ballard et al. 1993, Thomason et al. 1996, Long and Koob 1997, Meehan et al. 1997, Tullis and Peterson 1997).

The morphological development of the embryo is examined in chapter 2, with the production of a staging table suitable for this species. Several important morphological features and their relevance in development of the embryo are discussed. Series for developmental stages have already been described for most vertebrate groups and are useful as guides to developmental progress. However, it was not until Ballard et al. (1993) produced a series for normal development of the Lesser Spotted Dogfish (*Scyliorhinus canicula*) that a reasonably complete description of development from

first cleavage to hatching in elasmobranchs had been documented. Prior to this, descriptions often omitted developmental stages (Clark 1926, Baranes and Wendling 1981, Gilmore et al. 1983, Natanson and Cailliet 1986, Yano 1992), were a combination of several species (Balfour 1878) or lacked a time frame around which morphological features appeared (Smith 1942), making them poor comparative tools. Many of those studies that had missing stages were describing viviparous species with long gestation periods. This led to difficulties in getting enough samples throughout gestation for complete descriptions, because sampling was largely opportunistic. The study of embryonic development is much easier with oviparous species. The greater availability of eggs, which can be incubated in the laboratory under different environmental conditions, allows a greater understanding of development.

Ballard et al. (1993) had the advantage of being able to collect thousands of eggs and produced extremely detailed descriptions of early development. However they failed to adequately describe development in the 3 month period between capsule opening and hatching. During this time there is tremendous growth and morphological change, at least in *H. portusjacksoni*. The number of eggs collected in my study was limited and early descriptions are not as detailed as Ballard et al (1993), however later development is described in much greater detail in this study.

With the exception of oxygen and inorganic nutrients, oviparous elasmobranch embryos are supplied with all the nutrients necessary to complete development, making them excellent systems for the study of growth and energy metabolism. The rate of yolk utilisation, growth and incubation period in fish are temperature dependent (Rajagopal 1979, Herzig and Winkler 1986, Pipe and Walker 1987, Heming and Buddington 1988, Miranda et al. 1990, Pepin 1991) and this study examines these parameters at three temperatures in chapter 3. The effect of hastened incubation at higher temperatures on the size of teleost hatchlings is inconclusive and to date has not been examined in elasmobranch studies. Growth studies on embryonic elasmobranchs are uncommon (Clark 1926, Gilmore et al. 1983, Natanson and Cailliet 1986, Pelster and Bemis 1991, Tullis and Peterson 1997), and are conducted at only one temperature. This study examines the effect of temperature on growth in embryonic elasmobranchs for the first time.

The energetics of development are discussed in chapter 4. The energetics of embryonic development has been extensively studied in birds, and to a lesser extent, reptiles. In spite of these studies, there still remains controversy over basic physiological questions, such as the relationship between the length of incubation period and the energetic costs of development or the efficiency with which energy is transferred into animal tissue. Avian developmental costs were reported to be similar in precocial and altricial species, despite differences in incubation period, largely due to increased initial energy allotment in the longer incubating species (Carey et al. 1980, Ar et al. 1987). Incubation periods in most reptilian species are much longer than birds and are likely to incur greater maintenance costs and may result in a lower energy content of the hatchling. Compared to Aves, energy efficiencies were lower in the lizard, *Iguana iguana*, because of increased costs of maintenance (Ricklefs and Cullen 1973). Incubation in elasmobranchs is even longer than in reptiles, and maintenance issues are potentially even more important. Incubating elasmobranch embryos at different temperatures to assess maintenance costs has not been done to date, this study offering great advancement in comparative energetics. The ability to manipulate the incubation period in reptiles and fish by altering temperature, or by investigating a wide variety of bird species that have varying incubation periods allows the question of maintenance costs to be examined. This study was designed to create an energy budget for *H. portusjacksoni* incubated at 18, 20 and 22°C. The relative costs of development and respiration, and the efficiency of energy transfer from yolk to hatchling at each temperature was measured to assess whether longer incubation periods incur greater maintenance costs at the expense of growth. The changes in energy distribution of egg components as development proceeded was also examined.

Embryonic respiration and ventilation are presented in chapter 5. The early embryo primarily uses cutaneous respiration, branchial respiration becoming more important later in development (Kirshch and Nonnotte 1977, Lomholt and Johansen 1979, Feder and Burggren 1985, Steffenson and Lomholt 1985, Rombough 1989, 1998 and Takeda 1990). Oviparous elasmobranch development occurs in a sealed egg capsule for the first 40% of incubation, after which the capsule becomes open to seawater. Respiration in the early embryo is difficult to measure directly and this study presents morphological and physical data to describe early respiration. Appendix 3 describes the theoretical possibilities for aerobic metabolism during early development.

The measurement of the ontogeny of metabolism in older embryos is possible with open and closed respiratory systems. In avian embryos, the ontogeny of metabolism follows one of two general trends, based on the mode of development. Precocial embryos have a sigmoidal pattern of metabolic rate, while an exponential pattern is observed in the less advanced altricial embryo. Reptilian embryos also exhibit sigmoidal and exponential patterns. *H. portusjacksoni* hatches as a fully functional, miniature adult and would be considered precocial in avian terms, and likely to show similar growth and metabolism patterns.

Almost all metabolic and ventilatory studies on elasmobranchs have been conducted on adults at one temperature, the exceptions being Butler and Taylor (1975b) and Burton (1979). My study on *H. portusjacksoni* is the first to report on temperature effects on embryonic respiration in elasmobranchs, and one of two that examine the relationship between temperature and ventilation. The other study investigating temperature and embryonic ventilation was by Thomason et al. (1996). The effect of hypoxia on the respiration rate and ventilation in fish has been studied extensively in juvenile and adult fish, but embryonic forms have been neglected. This is surprising since it is the embryonic fish that are restricted in movement and cannot escape poor conditions. Oviparous elasmobranch embryos are occasionally subjected to periods of low oxygen within the capsule during development and this study describes the respiratory and ventilatory response to hypoxia.

Chapter 6 ties the previous chapters together for an overall understanding of embryonic development in *H. portusjacksoni*.

## Chapter 2 MORPHOLOGY AND COMPOSITION OF THE EGG AND EMBRYO

### 2.1 INTRODUCTION

#### 2.1.1 The egg and egg capsule

The eggs of oviparous elasmobranchs must be considered one of the largest among animal groups and are of great interest in terms of embryonic development. In both oviparous and viviparous elasmobranchs, the egg is surrounded by an outer capsule, which separates the embryo from the external environment for at least a portion of the development time. In the case of the viviparous embryo, the capsule is very thin (e.g. 4.7  $\mu\text{m}$  in *Mustelus canis* – Lombardi and Files 1993), and allows the exchange of small molecules between the uterine fluid and the embryo across the capsule membrane. In contrast, oviparous capsules are over 400 times thicker (up to 2 mm in *Scyliorhinus canicula* - Knight and Hunt 1976, Knupp and Squire 1998), consistent with an existence outside the female's body, where the incubation environment is much harsher and the embryos require greater protection. The capsule also functions to protect the developing embryo from predators and epibionts (Thomason et al. 1994). Antifouling on the capsule of the dogfish, *Scyliorhinus canicula*, due to a microbial biofilm, is effective against macrofouling organisms but not microbial foulers. By preventing or reducing the number of epibionts, the oxygen diffusion capabilities of the capsule would remain relatively uncompromised.

#### 2.1.2 Staging of *H. portusjacksoni*

The development of embryos within the egg environment can be described by a staging table. The process of defining particular stages within the continuum of embryonic development allows researchers to estimate the age of embryos against a set of known standards. Each stage is characterised by the appearance or morphological change of certain developmental features. There are many characters that can be used in staging a species, but if the staging is to be made useful for inter- and intra- specific comparisons, the characters must be relatively conservative. Ideally, a complete stage series should encompass the development from fertilisation to the production of a neonate (Billet et al. 1985). However there are inherent problems in trying to achieve this, such as the retention of eggs in the female's reproductive tract where some degree of development may occur and observation becomes difficult (Ewert 1985, Whitehead and Seymour 1990,

Ballard et al. 1993). Developmental diapause occurs in several reptilian species (Clark 1953, Dmi'el 1970), creating difficulties in comparing timing of the appearance of developmental stages.

Developmental stages in teleost fish species have been described by many authors (e.g. Ballard 1973, Crawford 1986, Galman and Avtalion 1989, Kimura et al. 1989, Hioki et al. 1990, Masuma et al. 1990, Kitajima et al. 1991, Munehara et al. 1991, Umezawa et al. 1991). These are useful in identifying species from larval forms and for fisheries management programs. Light microscopy techniques have been used in the majority of teleost embryological studies, but some employed Scanning Electron Microscopy (SEM) to observe the morphology of embryonic development in the teleost, *Oreochromis niloticus* (Galman and Avtalion 1989). This method did not allow the observation of internal morphology, but it was useful in capturing cleavage stages and the morphogenesis of external organs.

There have been a number of descriptions of elasmobranch embryos (Balfour 1878, Scammon 1911, Shann 1923, Clark 1926, Smith 1942, Te Winkel 1950, Baranes and Wendling 1981, Fujita 1981, Gilmore et al. 1983, Natanson and Cailliet 1986, Castro et al. 1988, Yano 1992, Ballard et al. 1993). Unfortunately these descriptions often involve only a few specimens and there is no attempt to stage embryos. There are often large developmental gaps between described embryos and in several studies, the early embryos are missing with descriptions beginning with already well-developed embryos. In contrast, others describe the early development in detail, but fail to include all the relevant information in older embryos. These descriptive narratives of elasmobranch development are reasonably well detailed but often lack information regarding the functional significance of various structures observed.

Some studies, however, are exceptional in their morphological detail. The early work by Balfour (1878), Scammon (1911) and Dean (in Smith 1942) described the development of elasmobranch fishes in great detail. Balfour documented general elasmobranch development using several species of sharks and rays. Although this may be useful in describing a continuum for elasmobranch development, it is not species specific. In 1942, Smith produced a document describing the natural history and external development of the oviparous Japanese shark embryo, *Heterodontus japonicus*, based on the 1906 work of Dean. From several hundred collected specimens, Dean detailed the morphological

changes that occurred over the 10 month incubation period, although neither he nor Smith attempted to attribute stages to embryos. More recently, an elasmobranch staging profile was carried out by Ballard et al. (1993) on Lesser Spotted dogfish embryos, *S. canicula*. This study describes early development in great detail, concentrating on gastrulation stages and development that occurs prior to capsule opening (or pre-hatching). Unfortunately, there is little discussion on morphology of stages between pre-hatching and hatching, a period lasting approximately 3 months.

Using some of Dean's criteria, I have produced a staging procedure for the Australian Port Jackson shark, *H. portusjacksoni*. Where data were missing, I have incorporated some of Dean's descriptions. I examined both live and dead material to determine the functional significance of morphological features. In particular, I directed attention towards the importance of circulation within the yolk membrane and embryo, rhythmic lateral movement of the embryo and the appearance of external gill filaments as respiratory aids for the young embryo. The possible function of the albumen and the significance of the opening of the egg capsule was examined, and I tested the hypothesis that the timing of the dissolution of the mucous plugs, and hence capsule opening, is initiated by the embryo.

The function of albumen in oviparous elasmobranch eggs has been hypothesised as both mechanical and osmotic protection to the early embryo (Smith 1936, Price and Daiber 1967). In order to assess the osmotic role of albumen in *H. portusjacksoni*, I measured the total osmotic and chloride ion concentrations in both yolk and albumen in freshly laid eggs and compared it to the surrounding seawater, with the aim of determining which membranes are responsible for osmoregulation.

Here I also describe the effect of ambient temperature on the timing of appearance of particular stages of *H. portusjacksoni*. The importance of incubating temperature on the state of developmental features in ectotherms is crucial for comparative purposes (Yntema 1968, Ballard 1973, Johns and Howell 1980, Deeming and Ferguson 1989). Yet many authors fail to consider this when working with ectothermic embryos, thus reducing the usefulness of their work. Under conditions of constant and known temperature, the occurrence of a particular stage should allow inference of the rate of developmental processes occurring for that species.

### **2.1.3 The structure of the elasmobranch gill**

Elasmobranchs commonly have five pairs of gills. The first (hyoid arch) bears one hemibranch while the remaining arches bear two hemibranchs located either side of the inter-branchial septum. Each inter-branchial septum is supported by cartilaginous rays and large constrictor muscles (Cooke 1980, Donald 1989). Along the length of each hemibranch are the primary gill filaments, which radiate dorso-laterally and ventro-laterally. Each filament bears a series of transverse plates called secondary lamellae, where gas exchange occurs. The secondary lamellae are thin-walled and consist of a double sheet of epithelium separated by the occasional pillar cell. The pillar cells form a support around the blood channels, which perforate the secondary lamellae. The extensions of the pillar cells form the lining of the blood sinuses (Bone and Marshall 1982).

#### **2.1.3.1 Blood flow through the gills**

There are two distinct but interconnected vascular systems within the elasmobranch gill: the systemic (or arterio-arterial) and the arterio-venous circulations. The systemic system supplies the secondary lamellae, while the arterio-venous circulation supplies the primary lamellae with a series of nutrient vessels and complex sinuses (Laurent and Dunel 1976, Randall 1982a, Al-Kadhomiyy 1984, Nilsson 1986, Butler and Metcalfe 1988, Laurent 1989).

The vasculature of elasmobranch gills is examined in this study using micro-vascular casting and scanning electron microscopy. Microvascular casting techniques allow a detailed study of the anatomical structures of the vascular system and the relationship between such structures (Gannon 1979, 1981, Gannon et al. 1982, 1983). This method of examination has been used in several studies relating to adult elasmobranch gill structure (Cooke 1980, Olson and Kent 1980, Donald 1989), where the unique vasculature of the elasmobranch gill was demonstrated. A complex system of non-respiratory capillaries appears to serve a nutritive function (Cooke 1980, Olson and Kent 1980) and the possibility of a respiratory by-pass shunt pathway was examined by Cooke. Within the elasmobranchs, there are slight variances of the vascular pathway, as observed in the two stingaree species examined by Donald (1989), however, in general the elasmobranch gill vascular system fundamentally resembles the teleost system.

Using the technique of microvascular casting, the relationships between blood vessels can be revealed.

### **2.1.3.2 Respiratory surface area**

Early embryos use their entire body surface as a respiratory organ (Fry 1957).

Cutaneous exchange predominates over gill respiration while the surface area is favourably high (Rombough and Moroz 1990, 1997, Rombough and Ure 1991, Wells and Pinder 1996b). As the embryo grows, however, the surface area may become limiting in its ability to facilitate oxygen uptake and other surfaces resume the respiratory role (Hughes and Al-Kadhomy 1988). These include the yolk-sac which is highly vascular (Rombough 1989), median and pectoral fins which extend the available area for exchange (Cunningham 1932) and the gills (Wells and Pinder 1996b, Rombough and Moroz 1997). During embryonic development in fish, it is common for unusual structures such as elongate fin rays, tentacles, eyestalks, trophotaeniae and external filaments to form, only to be lost later in development (Ballard 1973, Blaxter 1988). Trophotaeniae are projections of the hindgut that facilitate the transfer of maternal nutrients to the embryo in viviparous Goodeid fishes (Schindler and Hamlett 1993, Kokkala and Wourms 1994, Hollenberg and Wourms 1995). The equivalent in viviparous placental elasmobranch embryos is the appendiculae which are found on the umbilical cord (Castro and Wourms 1993). These structures effectively increase the surface area of the developing embryo, in order to facilitate nutrient transfer and enhance respiratory surface area.

Many fish embryos develop external gill tufts extending from the internal gill clefts out through the external gill openings (Fry 1957). External tufts greatly increase the surface area for gas exchange; they are thin-walled and vascular. As the fish further develops, these tufts can lengthen into filaments. The morphology of external gill filaments can be modified by environmental conditions, for instance the number and length can be increased or decreased depending on ambient oxygen availability (Løvtrop and Pigon 1968).

During the latter part of development in fish, the gill becomes the predominant respiratory organ. The secondary lamellae are the sites of respiratory gas exchange in the gill, and blood flow through them can be altered in order to change gas exchange dynamics (Soivio and Tuurala 1981, Randall 1982). The surface area of the gill varies

considerably between species, depending on the mass of individuals and the degree of activity of the fish. More active fish tend to have greater gill area per gram of body mass than less active ones (Fry 1957). The total surface area of the gills increases with mass to a power of 0.8-0.9 in several teleosts examined (Muir 1969, Rombough and Moroz 1997). Price (1931) studied the relationship between gill area and size of individuals and found that both the filaments and lamellae increase in size and number as the fish develops. He also found that the greatest change in the number of lamellae per unit length of filament occurred early in development.

## **2.2 MATERIALS AND METHODS**

### **2.2.1 Examination of eggs and egg capsules**

Eggs were collected from two sites in South Australia (Appendix 1, section 2.1, p181). A total of 138 eggs were weighed at the laboratory immediately following collection. The total lengths of 21 capsules were measured to  $\pm 1$  mm. The volume of 11 empty egg capsules was measured by filling them with seawater, and allowing it to drain into a tared beaker. The mass of water was determined and the volume established (1.025 g seawater = 1 ml seawater). This was repeated three times for each egg capsule, and a mean internal volume for each egg capsule was calculated.

The contents of nine freshly laid eggs were examined and the components weighed ( $\pm 0.1$  g) on a Sartorius 1265 MP balance. The eggs were opened by cutting the top end of the capsule off with a razor blade. The contents were gently poured into a 20 cm tared glass petri dish. Albumen was individually collected with 20 ml plastic syringes, emptied into tared containers and weighed. The yolk and the wet egg capsule were similarly weighed. The albumen from 16 eggs was separated as thick or thin fractions depending on the consistency. These albumen samples were weighed individually to examine the changes in proportionality as incubation proceeded.

### **2.2.2 Osmotic tension of the albumen and yolk**

#### **2.2.2.1 Total osmotic pressure**

The total osmotic pressure (mOsmol/kg) of albumen and yolk was measured in seven eggs with a vapour pressure osmometer (Wescor 1500C). The osmometer was calibrated using standard solutions supplied. From each fresh egg, a 50  $\mu$ L sample of heterogenous thin albumen was collected directly from the petri dish (2.2.1) with a glass capillary tube and diluted 1:1 with triple distilled water. Three 20  $\mu$ L sub-samples of the diluted albumen were collected with capillary tubes for analysis. The yolk was too viscous to be drawn up into capillary tubes, therefore a dilution was carried out by weight. A sample of yolk (5 g) was diluted with 45 g triple distilled water (1:10), measured on a Sartorius 1265 MP balance. Three sub-samples (20  $\mu$ L) were removed and analysed. The results were multiplied by the dilution factor to measure the total osmotic pressure.

### **2.2.2.2 Chloride concentration**

The total concentration of chloride ions ( $\text{mmol L}^{-1}$ ) was measured in albumen and yolk of seven eggs with a chloride titrator (Radiometer CMT10). Calibration was carried out with the standard solutions. Three samples of undiluted thin albumen and diluted yolk (1:10 from above) from each fresh egg were analysed.

### **2.2.3 Rate of egg capsule mass loss**

An estimation of the natural rate of deterioration (as measured by mass loss) of egg capsules was made on eleven empty egg capsules. The capsules were cut almost in half and the contents (yolk plus unattached albumen) removed. There were placed in aquaria at  $20^{\circ}\text{C}$ . The drained capsules were weighed in air every 25-28 days for a period of 107 days. The experiment began when capsules were already four weeks old. The rate of mass loss was determined as the difference between weight measurements as a function of time (grams per day). The thickness of the egg capsule around the cut edge was determined ( $\pm 0.1$  mm) over the same 107 day period. Measurements were made possible by squeezing the centre of the egg capsule to expose the slit edge. Callipers were used to measure the thickness at three locations around the edge and an average value was determined.

### **2.2.4 The effect of the embryo on timing of egg capsule opening**

The hypothesis that the embryo initiated the opening of the egg capsule was tested using both 'experimental eggs' (those with intact plugs and undisturbed contents, that is, healthy developing eggs) and 'empty' egg capsules (with intact plugs but emptied of the contents) at three temperatures. A total of 48 experimental eggs were distributed in three aerated, filtered aquaria at 18, 20 and  $22^{\circ}\text{C}$ . The approximate date of laying was known for each egg (Appendix 1, section 4.4, p.192). These eggs were allowed to develop normally and were used for other experiments after egg capsule opening was observed. Fourteen egg capsules were opened prematurely by cutting a small slit (approximately 3 cm long) in the side and squeezing out the contents. The albumen was removed with the exception of that directly attached to the plugs. The egg capsules were then washed thoroughly in fresh sea water so that all yolk remains were removed. The egg capsules were filled with sea water and placed in aquaria at  $20^{\circ}\text{C}$ .

The opening of the egg capsule can be determined in two ways. When the egg is lifted out of water, the fluid inside the egg capsule drains through the open slits.

Alternatively, the flanges forming the lip at the broad end can be twisted in opposite directions to expose the inside of the egg capsule (Figure 2.1). While the plugs are present this is not possible. Eggs were checked twice weekly for evidence of opening. The experimental capsules with live embryos were also monitored for opening dates.

I observed the pattern of mucous plug deterioration from five egg capsules that were cut completely in half, thus exposing the mucous plugs. The ten half-capsules were cleaned, and placed into aquaria at 20°C. Photographs were taken at 0, 2 and 3 months to demonstrate the pattern of loss of the mucous plug.

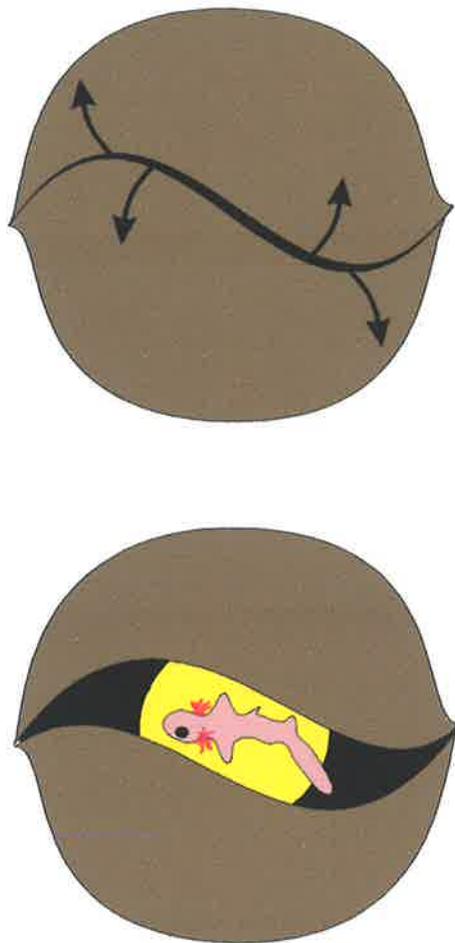


Figure 2.1 The opening of the egg capsule is determined when the broadside flaps can be drawn apart and the egg contents viewed.

### **2.2.5 Staging procedure**

A total of 60 eggs were chosen from each of three temperature regimes (18, 20 or 22°C) at selected times throughout incubation and killed to allow examination of the embryos. Each sample consisted of three eggs, which were initially estimated to be of similar age (Appendix 1, section 4.4, p. 192). This was inferred from date of laying (within 2-4 weeks) and verified by morphological similarities between the embryos. Embryos that were obviously of a different age were still examined but not used for staging.

The embryo was exposed by carefully removing the top third (broad end) of an egg capsule with a razor blade. As the embryo sat on top of the yolk, observations of morphology and activity could be easily made. The embryo was then photographed and killed by adding a lethal dose of anaesthetic tricaine methanesulphonate (Sandoz MS-222) to one litre of seawater (ca. 1g/g of embryo). The embryo was separated from the yolk sac by cutting the yolk stalk with scissors and placed into a tared plastic petri dish. Morphological features were drawn and the total length was recorded with callipers. The wet masses of embryo, yolk and albumen were recorded to the nearest mg on a Sartorius 1265 MP balance. Once weighed whole, the embryos were dissected and any internal yolk was removed and weighed.

### **2.2.6 Gill vasculature**

#### **2.2.6.1 Microvascular casting method**

The microvascular casting technique used follows that of Gannon (1979, 1981). Three embryos (stage 11-12) possessing varying degrees of external gill development ranging from extensive external development (10 mm long filaments), to no external development, were chosen to examine the external and internal gill vasculature. Each embryo was removed from the egg capsule and placed into a dish of seawater. It was immobilised by the addition of MS-222 to one litre of seawater (ca. 10 mg/g of embryo). Once immobile, the umbilical vein in the yolk stalk was exposed and cannulated with vinyl tubing (OD 0.5 mm) which was attached to a needle and 1 ml syringe partly filled with saline. This vessel was chosen in preference to the heart because of the difficulty in exposing the heart vessels without damaging the closely associated gill vasculature.

When the cannula was in place, the plastic was mixed. The plastic used was the monomer methyl methacrylate. In its original form, the monomer was a fluid and needed to be polymerised by mixing with a hydroxy propyl methacrylate monomer and an initiator compound. Once mixed, the fluid plastic was drawn into a 2 ml syringe that then replaced the 1 ml syringe attached to the cannula. The plastic was slowly injected into the circulatory system of the animal over a period of 1 min. To prevent pressure build up and subsequent vessel damage, the caudal vessel was cut to allow an outflow. Approximately 1-1.5 ml of plastic was injected and the animal left while the plastic hardened in the vessels, creating the vascular cast.

The cast was then freed from the animal tissue surrounding it by immersion in 30% KOH and subsequent and repetitive daily washings in hot tap water and then returned to a 15% KOH solution. This was done very carefully as the cast was fragile. The cast was placed in a metal basket to reduce handling. This process took 2-4 weeks to complete.

The casts were then cleaned thoroughly in an ultrasonic cleaner in distilled water with a small amount of detergent for 10-20 min and washed in distilled water several times before being dried. The casts were air-dried in a dust free area and placed onto specimen stubs, coated with gold/palladium and viewed in an ETEC scanning electron microscope.

## 2.3 RESULTS

### 2.3.1 The egg and egg capsule

A freshly laid egg weighs  $155.5 \pm 7.9$  g (mean  $\pm$  SE) (n=138) (range 90.1-208.1 g) and is  $128.6 \text{ long} \pm 2.4$  mm (21) (range 105-180 mm). The internal volume was  $121.8 \pm 9.4$  ml (11). The mean thickness of a fresh egg capsule was  $1.02 \pm 0.521$  mm (14) (range 0.49-1.52 mm). There are two respiratory slits, one at either end of the capsule. When the egg is first laid, these slits are plugged up by a thick plug of mucous (known as a hydrated mucopolysaccharide - Thomason et al. 1994) on the inside (Figure 2.2). Later (4 months) they dissolve and the capsule becomes open to seawater (Figure 2.3).



Figure 2.2 The respiratory slit at the broad end of the capsule is sealed by a thick S-shaped plug of mucous.

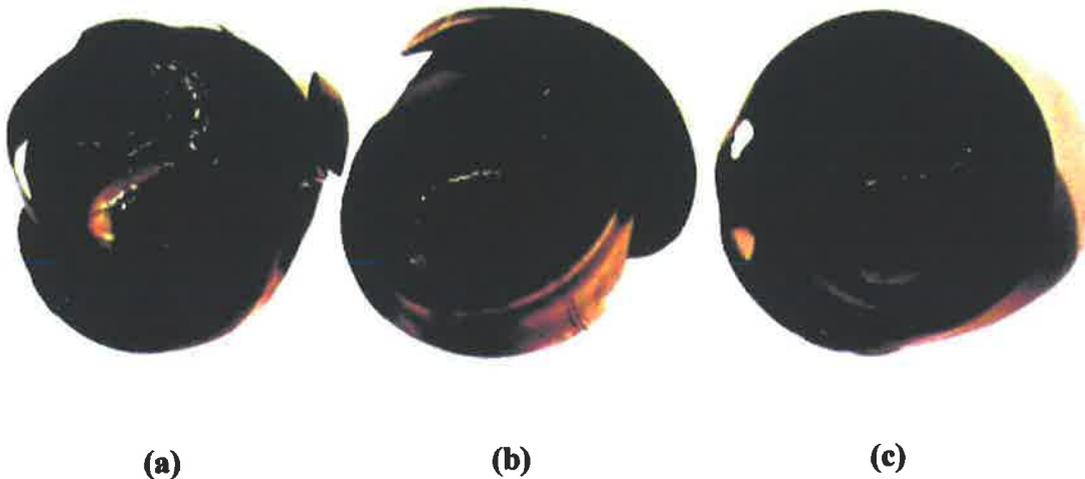


Figure 2.3 The mucous plug dissolves gradually over time. a = 0 months, b = 2 months and c = 3 months.

The capsule is surrounded by a double helix flange, which originates at the broad end of the capsule and winds around the capsule 2-3 times, before ending at the narrow end (Figure 2.4).



Figure 2.4 An egg capsule of *H. portusjacksoni*. The arrow points toward the position of the broad-end respiratory slit. Scale = 10 mm.

The egg contains a large yolk, surrounded by albumen (Figure 2.5). The yolk is not attached to the egg capsule but floats in the thin albumen. Each egg capsule contains only one embryo.

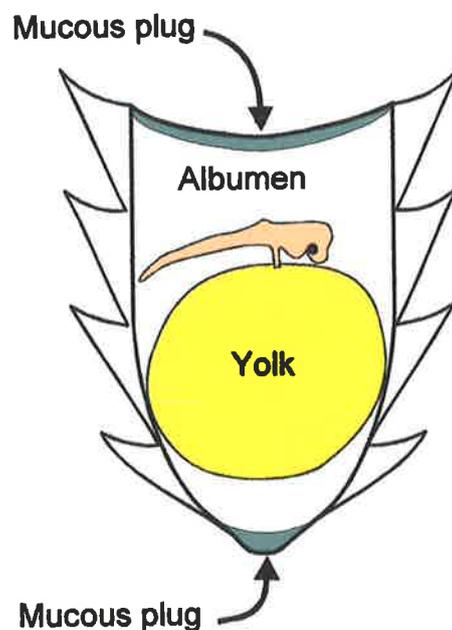


Figure 2.5 A cross-section of the egg shows the relative positions of the yolk, embryo and albumen. The ends of the capsule are plugged with a mucous plug during the first four months of incubation.

The contents (as mean mass and percentage of the egg and of total contents) of egg components from nine fresh eggs is presented in Table 2.1.

<b>Component</b>	<b>Mean <math>\pm</math> SE</b>	<b>Range</b>	<b>% egg</b>	<b>% contents</b>
<b>Total mass (g)</b>	170.2 $\pm$ 5.01	147.6 - 188.3	100	
<b>yolk</b>	38.6 $\pm$ 1.28	30.1 - 42.8	22.7	35.2
<b>total albumen</b>	70.7 $\pm$ 2.78	56.2 - 87.9	41.5	64.8
<b>egg capsule</b>	60.9 $\pm$ 3.15	48.6 - 81.3	35.8	

**Table 2.1** Mass (g) and percentages of initial egg components of nine fresh eggs. Results are mean  $\pm$  standard error. Note that the total mean mass (170.2 g) was a sub-sample of the total number of eggs collected, where the mean egg mass was 155.5 g. The eggs chosen for the sub-sample were overall larger than the total sample.

The albumen can be partitioned into two types. There is a thick viscous albumen, which originates from the mucous plugs found at either end of the capsule and occupies the area closest to the capsule. The other type of albumen is much thinner and surrounds the embryo. As development proceeds, the percentage of thick albumen decreases while the percentage of thin albumen increases (Table 2.2). At 120 days the mucous plugs have dissolved, the slits open and the embryo and yolk are surrounded by seawater.

### **2.3.2 Osmotic concentration of yolk and albumen**

Thin albumen is slightly hyper-osmotic to seawater and to the plasma of juvenile sharks (Table 2.3), whereas the yolk is hypo-osmotic. A large osmotic component of seawater is NaCl as indicated by the high chloride concentration (49%). Similar proportions of chloride are found in the albumen (48%), confirming a high inorganic make up of albumen, and a marked similarity to seawater. In comparison, the yolk has a low chloride component (16%), hence NaCl makes up only as much as 32% of the total osmotic concentration. The remaining yolk components are likely to be the organic compounds urea and tri-methyl amine oxide (TMAO), important osmoregulatory substances for elasmobranchs.

age (days)	% of total contents	
	thick albumen	thin albumen
15	37.3 ± 6.60 (3)	24.7 ± 3.51 (3)
45	38.7 (1)	26.8 (1)
60	39.6 (2)	23.2 (2)
75	31.2 (1)	33.2 (1)
90	18.2 (2)	39.8 (2)
105	10.6 ± 1.88 (4)	54.5 ± 3.16 (4)
112	5.3 (1)	58.8 (1)
120	0 (2)	0 (2)

**Table 2.2** Percentage of thick and thin albumen of total contents from 16 individual eggs during the first four months of incubation. Where the sample size is greater than three, data are mean ± standard error (n). Where the sample size is fewer than three, only the mean (n) is provided. At 120 days the capsule opens and there is no albumen present in either form.

	Total osmotic concentration (mOs kg <sup>-1</sup> )	chloride concentration (mmol L <sup>-1</sup> )
yolk	676.6 ± 11.31 (7)	92.9 ± 12.19 (6)
albumen	1202.4 ± 29.42 (7)	573.7 ± 22.59 (7)
plasma	1148	306
seawater	1141.5	558.5

**Table 2.3** Total osmotic concentration and chloride concentration of yolk and albumen from fresh eggs. Data are mean ± standard error (n). The values for plasma are from juvenile *H. portusjacksoni* sharks measured by Withers et al. (1994), and seawater values are taken from Potts and Parry (1964) for a salinity of 35 g L<sup>-1</sup>.

### 2.3.3 Changes in mass and thickness of the egg capsule over time

Egg capsules lost approximately 30 g during the 107 days of observation. The rate of mass loss was initially high and gradually decreased with time until it stabilised (Figure 2.6). In the course of the experiment, the loss was not due to decreasing capsule thickness. Over the 107 day period there was no significant change in the thickness of the empty capsules (Figure 2.7) ( $T = 0.147$ ,  $P > 0.05$ ).

### 2.3.4 The size of embryo at egg capsule opening

Results from an ANOVA show that there was no significant difference in wet embryo mass at the three temperatures at the time of egg capsule opening ( $F = 2.858$ ,  $P > 0.05$ ). However, total embryonic length was significantly different, occurring between 20 and 22°C ( $F = 9.175$ ,  $P < 0.05$ ) (Table 2.4).

temperature	embryo wet mass (g)	total embryo length (mm)
18°C	0.927 ± 0.096 (2)	54.00 ± 3.08 (4)
20°C	0.710 ± 0.078 (7)	54.22 ± 1.51 (9)
22°C	0.437 ± 0.185 (3)	45.75 ± 2.49 (4)

**Table 2.4** Mean embryo mass and length at the time of egg capsule opening ( $\pm$  standard error (n)) at the three experimental temperatures.

### 2.3.5 The role of the embryo on egg capsule opening

The average date of opening ( $\pm$  standard error (n)) of laboratory incubated eggs was January 24 ± 5.9 (12), January 21 ± 2.3 (18), January 20 ± 3.1 (18) at 18, 20 and 22°C respectively. There was a large variance in the data, ranging from late December through to late February. There was no significant difference between opening dates of experimental eggs at the three temperatures; opening occurring approximately 120 days after laying ( $F(2,46) = 0.672$ ,  $P > 0.05$ ).

The average date of opening of the empty egg capsules was January 29 ± 4.28 (14). There was no significant difference between opening dates of experimental and empty egg capsules at 20°C (two sample T-test,  $T = -1.55$ ).

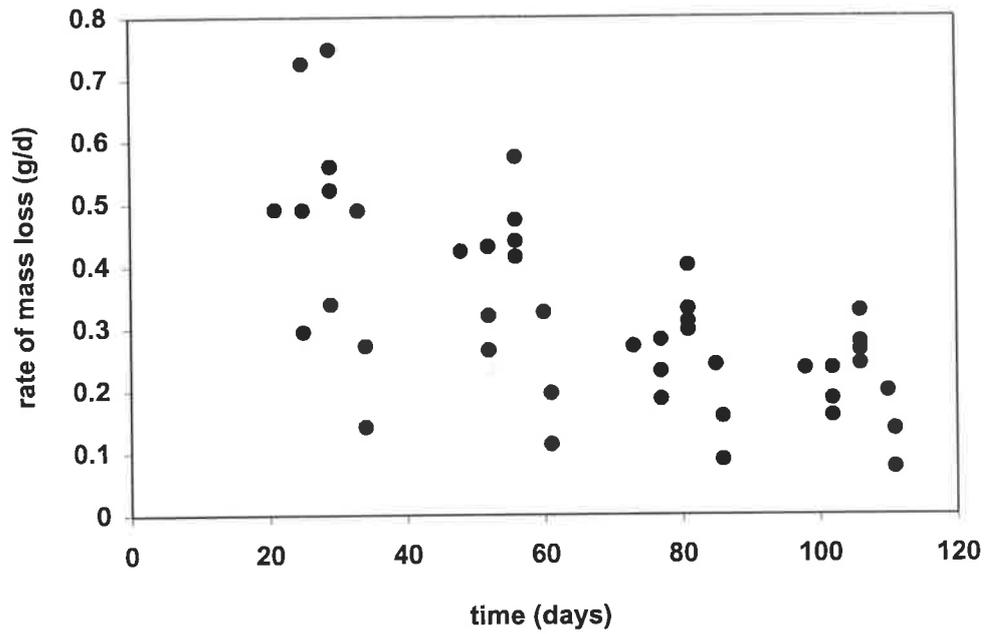


Figure 2.6 The rate of egg capsule mass loss (g/d) during incubation. Each grouping of data points represents repetitive sampling of the same capsules over time.

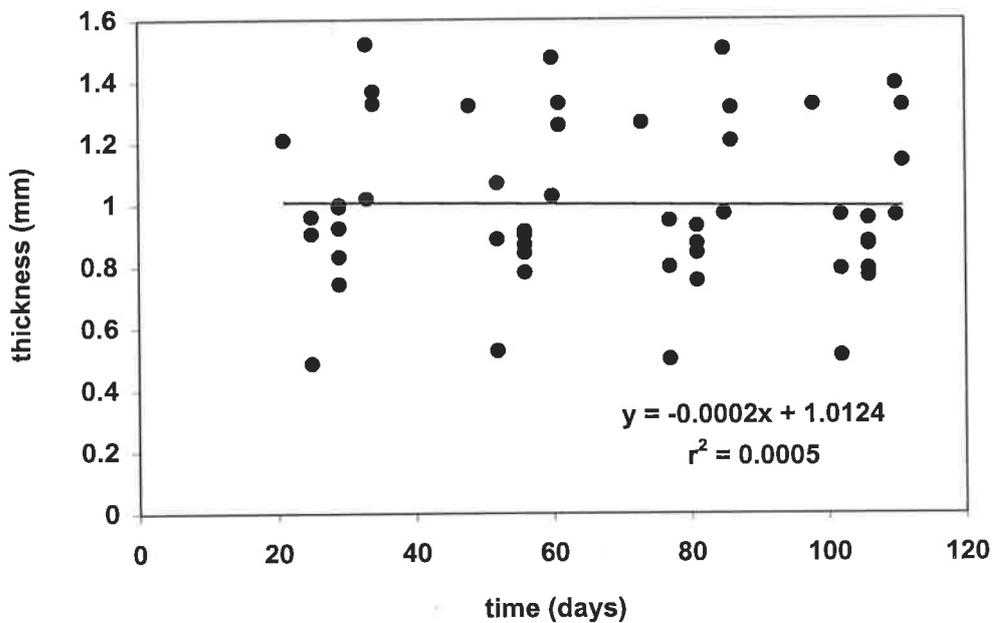


Figure 2.7 Changes in capsule thickness over time. Each grouping of data points represents repetitive sampling of the same capsules over time.

### 2.3.6 Staging table for *Heterodontus portusjacksoni*

The morphological development of *H. portusjacksoni* is described in the following staging table. The first ten stages involve morphological changes that occur within the sealed egg capsule, the remaining five stages describe development in the open capsule. All descriptions were based on data at 20°C. A summary is presented in Table 2.5.

Stages 1-3 describe the changes which occurred in the first month of external incubation.

#### Stage 1

The blastoderm (observed as an orange spot) appears on the upper surface of the yolk sac (Figure 2.8). This is composed of two types of cells: flattened surface blastomeres and an inner mass of deep blastomeres. The yolk is surrounded by albumen which is continuous with the mucous plugs at either end of the capsule.

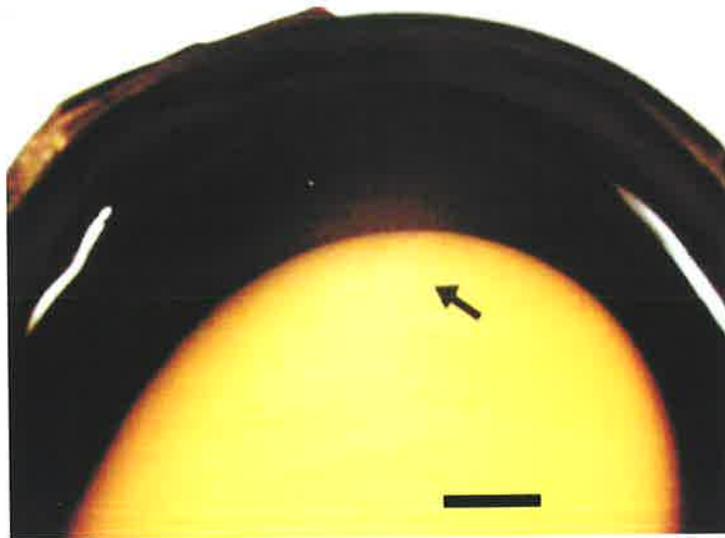


Figure 2.8 A blastoderm stage embryo (see arrow). Scale = 1 mm.

#### Stage 2

During gastrulation, neurulation commences with the first sign of development of the neural groove and neural folds. This stage was not observed in *H. portusjacksoni*, therefore I include text figure 43(b) of Smith (1942) as Figure 2.9 to demonstrate this stage of development.

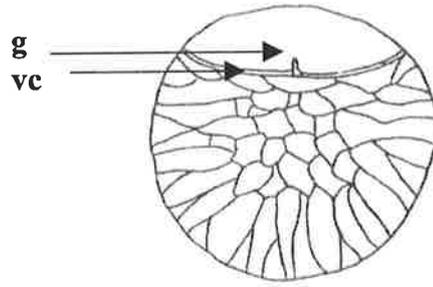


Figure 2.9 Gastrula stage (g) of *H. japonicus* from figure 43(b) in Smith (1942). The main branches of the vitelline circulation have formed (vc), the other lines in the yolk represent actual or incipient cleavage furrows.

### Stage 3

The development of cervical and cephalic flexures begins at this stage. The head is positioned away from the central axis of the body (Figure 2.10). The tip of the tail is unsegmented. Figure 22, plate II (Smith 1942) represents an accurate description of this stage.

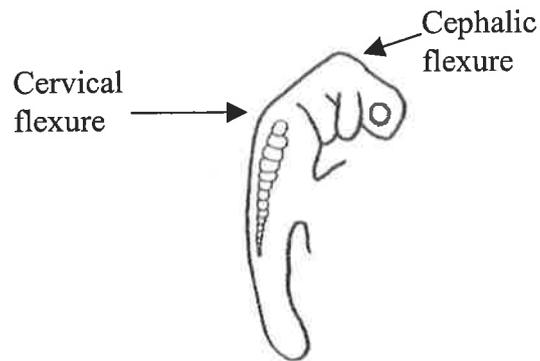


Figure 2.10 Cervical and cephalic flexures in the stage 3 embryo. From Figure 22, plate II (Smith 1942).

The second month of incubation is described by the stages 4 & 5.

### Stage 4

At six weeks, rudimentary otic and optic vesicles appear. The body becomes more arched, with the tail (now fully segmented) curled inwards. The first appearance of the gill slits is observed at this stage. Figure 2.11 represents an embryo with four gill slits, the fourth still very small. There is no obvious mouth opening yet. A notochord runs

the entire length of the body. The embryo-yolk connection is very large, with no discernible yolk stalk. The embryo is 5-10 mm long and is still transparent.

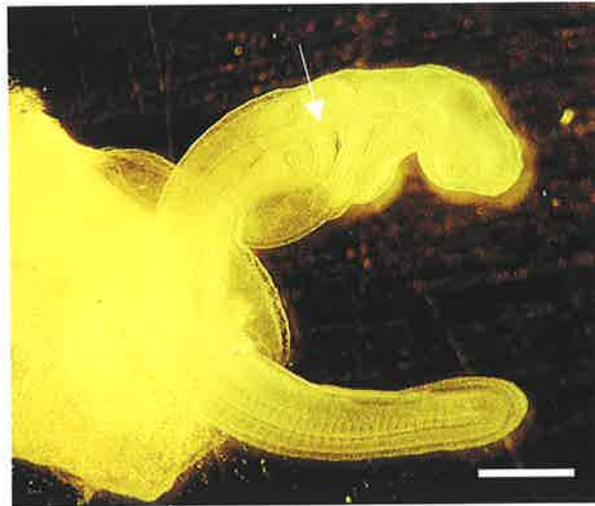


Figure 2.11 A stage 4 embryo showing the formation of gill slits (see arrow).  
Scale =1mm.

### Stage 5

At two months, the spiracular slits form anterior to the first branchial slit and posterior to the eye (Figure 2.12). The heart is first observed to be active and the main branches of the vitelline circulation are present. Definite rhythmic body movements are observed at this stage. The embryo-yolk connection is reduced, but still covers over half the embryo's length. The embryo is 5-15 mm long and has 50-60 somites. The optic vesicle advances upwards away from the rostral region.



Figure 2.12 A stage 5 embryo showing spiracular gill slit (s), heart region (h) and optic vessel (ov). Scale = 1mm.

## Stage 6

At three months, there is extensive yolk and cutaneous vascularisation (Figure 2.13). Blood vessels completely surround the yolk sac. The embryo becomes opaque and is 15-20 mm long. The first appearance of fin budding appears from the continuous fin fold enveloping the embryo. The pectoral fins develop first, followed by the two dorsals. The tail is still undefined in shape, bordered by a rounded fin fold. From this fin fold, the anal and caudal fins later emerge. The yolk stalk is now a small distinct connection located behind the heart.

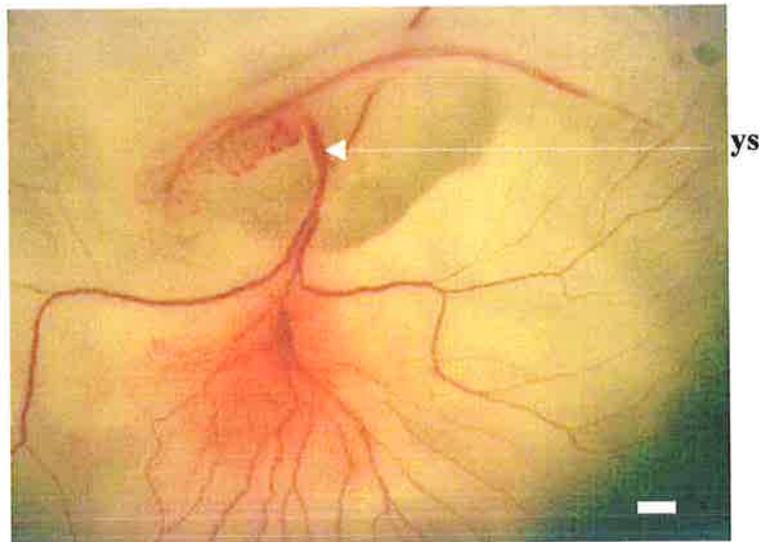


Figure 2.13 A stage 6 embryo with extensive vascularisation of the yolk and skin. The yolk stalk (ys) is located just behind the heart (see arrow). Scale = 1mm.

The yolk stalk can be seen to have various structures associated with it: yolk or vitelline artery, vitelline vein, and a central ductus vitello-intestinalis through which the yolk is transported (Figure 2.14). A cross section of the yolk stalk shows it is made of four layers. The central vessels (artery, vein, and ductus vitello-intestinalis) are surrounded by a cell layer. Around this is a coelomic fluid space which presumably is continuous with the coelomic cavity of the embryo. A jelly-like substance surrounds the coelomic cavity, and finally a relatively thick ectoderm covers the entire organ.

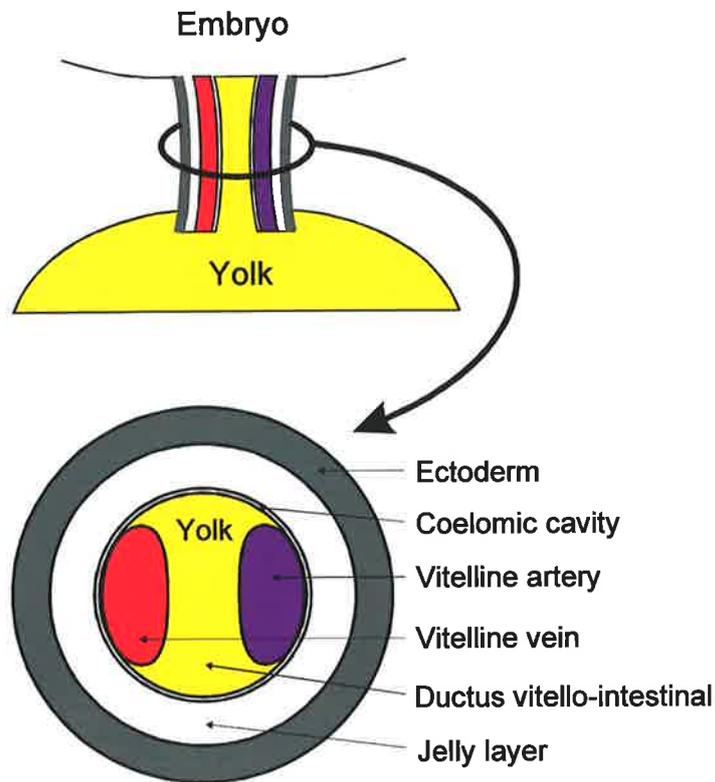


Figure 2.14 The yolk stalk is composed of several layers and vessels.

### Stage 7

During the third month of incubation, external gill filaments first emerge from the gill slits. These are initially visible as small tufts (Figure 2.15). They are first observed from the first gill slit, followed by the remaining gill slits. The spiracular filaments are the last to develop. Embryos are 20-30 mm long.

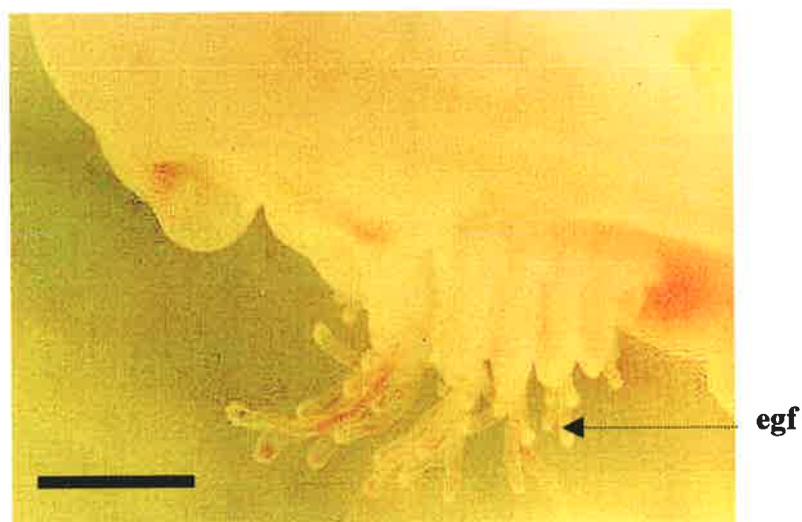


Figure 2.15 A stage 7 embryo showing the first appearance of external gill filaments (egf) with blood flowing through them. Scale = 0.5mm.

### Stage 8

Between 3 and 4 months, the axial fins take on a definite rounded shape. The caudal fin is now elongate. Retinal pigmentation begins to encircle the eye, beginning at the outer edge (Figure 2.16). The external gill filaments are longer and more numerous.

Embryonic length is 20-40 mm.

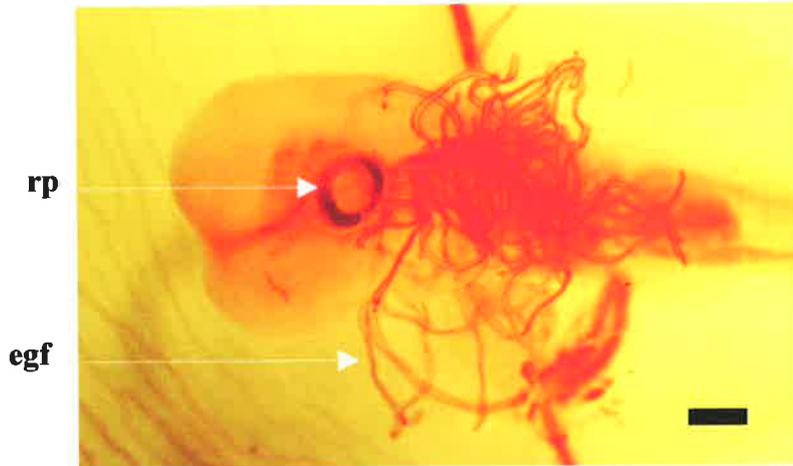


Figure 2.16 A stage 8 embryo demonstrating retinal pigment (rp) and extensive external gill filamentous growth (egf). Scale = 1mm.

### Stage 9

At four months, the mouth becomes recognisable. The bases of the dorsal fins become narrower, giving them a shape more typical of the adult form (Figure 2.17). The region from which the spines later emerge is clearly defined. Fin rays develop in all paired fins (Figure 2.18) and an indistinct lateral line can be observed. Pigmentation completely surrounds the eye at this stage. The embryo is 30-40 mm long.

### Stage 10

Still at four months, but later than Stage 9, the dorsal fin develops a spine that is characteristic for the genus (Figure 2.19). The pelvic and anal fins lose their rounded shape and assume a shape typical of the adult. External gill filament growth is extensive (up to 10 mm long). The first external appearance of sexual organs (claspers) occurs in the 40-50 mm long stage 10 male embryo.



Figure 2.17 The dorsal fin takes an adult shape in the stage 9 embryo. Scale = 1mm.

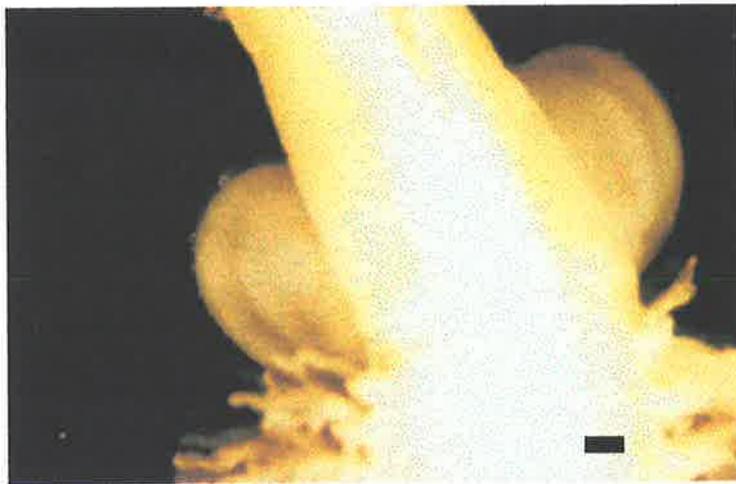


Figure 2.18 Fin rays develop in the paired pectoral fins at stage 9. Scale = 1mm.



Figure 2.19 The emergence of the dorsal spine in the stage 10 embryo. Scale = 1mm.

During the fourth month of incubation the mucous plugs dissolve, creating an open flow egg capsule. The embryo is 45-55 mm long at opening.

### Stage 11

At four-and-a-half months, the external gill filaments reach maximum development (12 mm long) in the 80-90 mm long embryo (Figure 2.20(a)). Resorption of the external gill filaments into the internal gill structure begins (Figure 2.20b). During this resorption, the internal gills first become functional. The supra-orbital ridge, characteristic of the species, becomes predominant at this stage. In the following sequence of figures (2.20), the scale = 10mm.

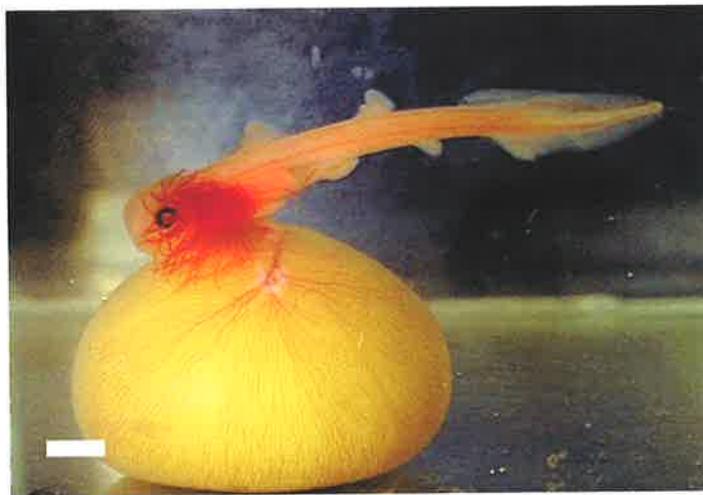


Figure 2.20a A stage 11 embryo with maximum external gill filament growth.



Figure 2.20b External gill filaments begin to be reabsorbed during stage 11.



Figure 2.20c Pigmentation of the body begins in the stage 12 embryo.



Figure 2.20d Reabsorption of the external filaments continues in the stage 12 embryo.



Figure 2.20e External filaments are almost fully resorbed by the end of stage 12.

### Stage 12

At five months, pigmentation of the body begins with a band across the supra-orbital ridge and over the eyes (Figure 2.20c). The external gill filaments continue to be reabsorbed back into the internal gill structure (Figures 20c-e). At five-and-a-half months, the external gill filaments are completely resorbed. The external yolk sac begins to be internalised in a sac attached to the anterior section of the spiral intestine (see Figure 2.22b for details on the internal and external yolk connection). The embryo is 120-130 mm long.

### Stage 13

By late in the fifth month, the embryo is extensively pigmented (Figure 2.21a). The head becomes larger with respect to the rest of the body with one consequence being that the distance between the spiracle and first gill slit increases. By the end of this period, the embryo resembles an adult with the exception of the external yolk which is still attached. The external yolk diminishes in size as internal stores grow (Figure 2.21b). Total embryonic length is 160-170 mm.



Figure 2.21a The stage 13 embryo is extensively pigmented with small external yolk reserves (ey).

### Stage 14

Between seven and eight months, the external yolk is completely exhausted, partly used for growth and in part internalised in a large internal sac (Figures 2.22 a & b) The internal yolk is attached to the anterior end of the spiral gut. Embryo growth is slow at this stage, with total length being 170-190 mm long.

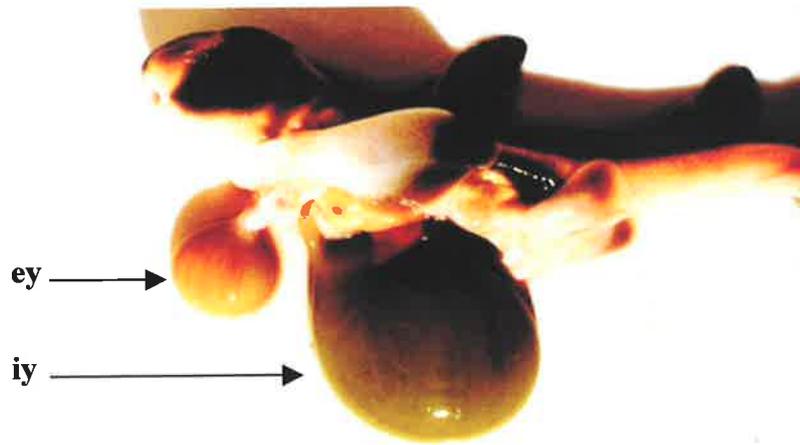


Figure 2.21b The internal yolk (iy) stores in the stage 13 embryo are large.

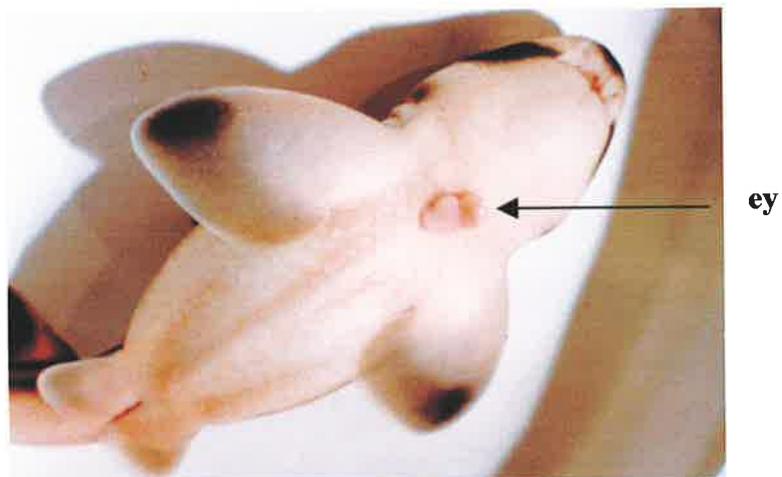


Figure 2.22a The external yolk (ey) is almost completely exhausted by stage 14.

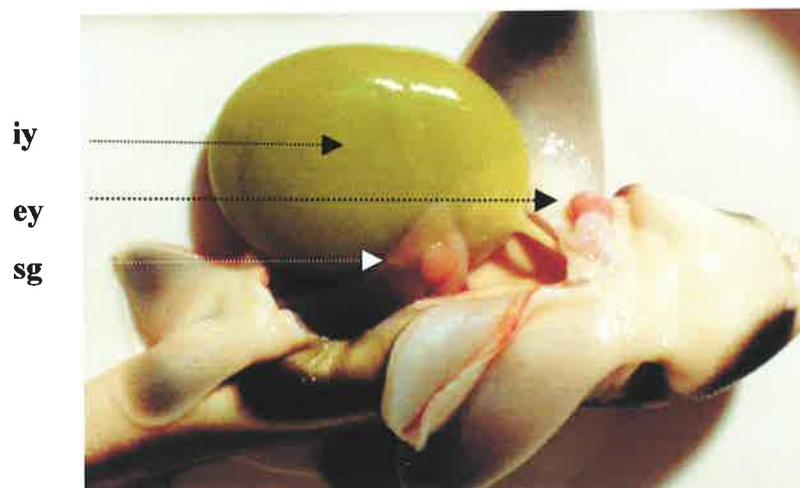


Figure 2.22b The internal yolk sac (iy) is large in the stage 14 embryo and is connected to the anterior of the spiral gut (sg).

## Stage 15

Internal yolk stores are virtually depleted (mean mass  $0.419\text{g} \pm 0.121$  ( $n=6$ )) and hatching occurs at 10-11 months. A small umbilical scar exists where the yolk stalk connection to the external yolk sac was located earlier in the incubation. Total length of hatchlings is 180-220 mm. Hatching in *H. portusjacksoni* is a rapid process, taking only seconds (Smith 1942, this study). The embryo's position within the capsule is limited by the internal dimensions of the egg capsule (mean volume = 121 ml). Prior to hatching, the embryo is curled around itself in the capsule. The nose is directed towards the narrow end of the capsule and the tail is wrapped around the top of the head (Figure 2.23a). Just prior to hatching, the embryo turns around such that the head is at the broad end of the capsule and the mid body is at the narrow end (Figure 2.23b). The embryo then "springs" out from its egg capsule head first through the broad end opening.

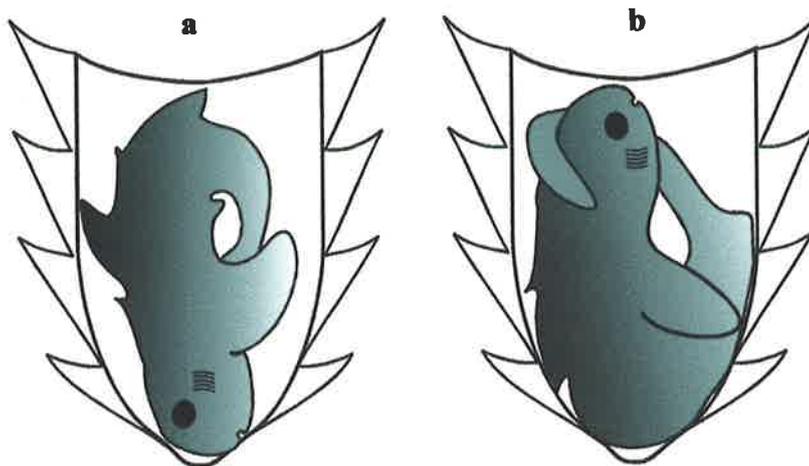


Figure 2.23 The position of the embryo well before hatch (a) and just prior to hatch (b).

### 2.3.7 Effect of temperature on developmental stages

The staging method used in this study describes development at  $20^{\circ}\text{C}$ . The number of embryos examined at  $18^{\circ}\text{C}$  and  $22^{\circ}\text{C}$  was limited but developmental stages observed at any point of incubation appear to be temperature dependent (Fig. 2.24). Embryos incubated at  $22^{\circ}\text{C}$  appear to be at least 1 month ahead of similarly staged embryos at  $20^{\circ}\text{C}$ . For example, cutaneous and yolk membrane circulation, rounded shaped fins (stage 6), external filament growth (stage 7) and eye pigmentation (stage 8) occur between 2-3 months at  $22^{\circ}\text{C}$ , compared to 3-4 months at  $20^{\circ}\text{C}$ . The initiation of body

stage	age (month)	total length (mm)	definitive character
1	0.5	0	blastoderm present
2	1	0	gastrula present
3	1.3	5	cervical and cephalic flexures
4	1.5	5-10	otic and optic vesicles present branchial gill slits present
5	2.0	10-15	spiracular gill slit present heart region active
6	3.0	15-20	yolk vascular pectoral and dorsal fins shaped
7	3.3	20-30	external gill filaments present
8	3.5	25-40	caudal fin shaped eye pigmentation begins
9	3.75	30-40	mouth recognisable dorsal fins narrow at base
10	4.0	40-55	dorsal spines present claspers present
11	4.5	80-90	external gill filaments maximum length (12 mm) filament reabsorption begins
12	5.0	120-130	external filaments absent body pigmentation starts
13	6.0	160-170	body pigmentation complete
14	7.5	170-190	external yolk absent
15	10.5	180-220	internal yolk absent hatching occurs

Table 2.5 Summary of staging table for *H. portusjacksoni* at 20°C.

pigmentation with the stripe across the eyes and of yolk internalisation, along with the complete resorption of external filaments (stage 12) occurs at approximately 4 months at 22°C, whereas similar features are not observed until the fifth month of incubation at 20°C. This developmental rate continues such that hatching occurs 1 month earlier at 22°C.

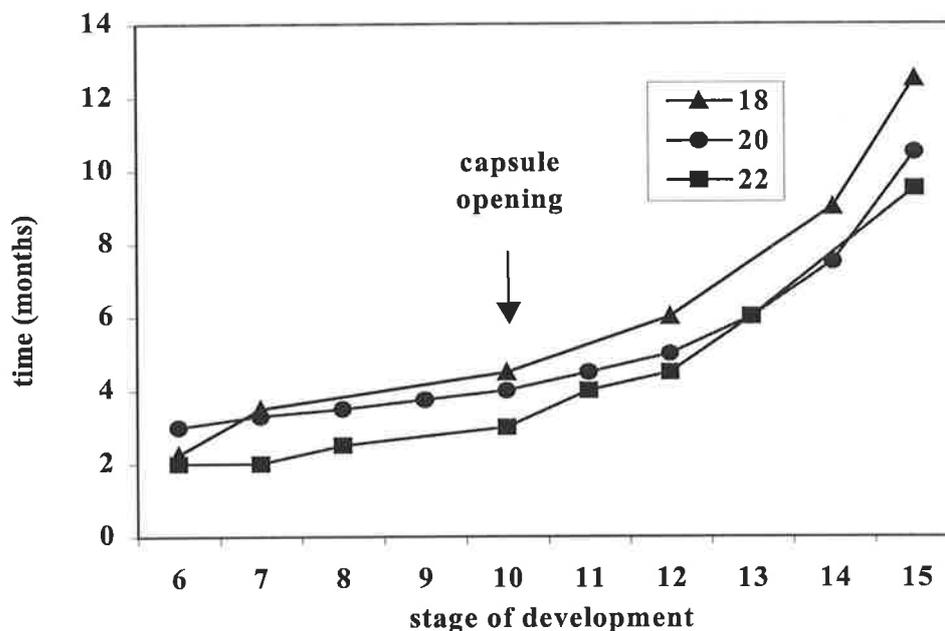


Figure 2.24 The effect of temperature on the timing of developmental stage at 18, 20 and 22°C.

Developmental features at 18°C appear to be 1 month behind similarly staged embryos at 20°C. The extent of fin development (stage 6), external filament growth (stage 7) and appearance of eye pigment (stage 8) appear earlier in the warmer incubated embryos. Stage 10 embryos with adult shaped fins are observed late in the fourth month of incubation at 20°C but not until the sixth month at 18°C. The initiation of yolk internalisation and complete resorption of external filaments in stage 12 embryos occurs at 5 months at 20°C and at 6 months at 18°C. The slowing of development at 18°C is even more exaggerated at hatching time where embryos hatch two months later.

### 2.3.8 Microvascular casting

The results are observed from photographs taken from a binocular microscope and from scanning electron microscopy (SEM) micrographs. Dorsally, the heart consists of a sinus venosum which leads into a large atrium; ventrally, the ventricle and conus are observed. The ventral aorta exits the conus of the heart as a single major vessel, which

immediately branches into two vessels each of which feed the gills on both sides. Each branch further divides to deliver blood to each of the five gills and the spiracle. From each individual gill vessel, a branchial artery enters each primary filament (Figure 2.25). From the afferent and efferent branchial arteries the afferent and efferent arterioles transport blood through the secondary lamellae. The afferent arterioles lead into a large sinus system (cavernous tissue). Blood is then transported to the secondary lamellae where gas exchange occurs. Oxygenated blood leaves the secondary lamellae through the efferent arterioles and into the efferent branchial artery (Figures 2.26, 2.27) where it is transported to the body via the dorsal aorta. The spiracle is fed by a vessel which branches off the main branch of the ventral aorta shortly after the primary branch to the gills. Each filament of the spiracle is individually fed by a branchial artery (Figure 2.28). The nutritive vessels associated with the primary filament are not obvious from the casts in this study.

The relationship between the internal and external gill vasculature is clear. The afferent and efferent arteries of each primary filament continue past the tip of the internal filament to form a blood-filled unattached capillary loop known as the external gill filament (Figure 2.29). However the continuous nature of this loop is not observed in this figure because of the fragile nature of the vessels which were broken during preparation of the cast. It can however be clearly observed in Figure 2.16 (p. 30) in a stage 8 embryo.

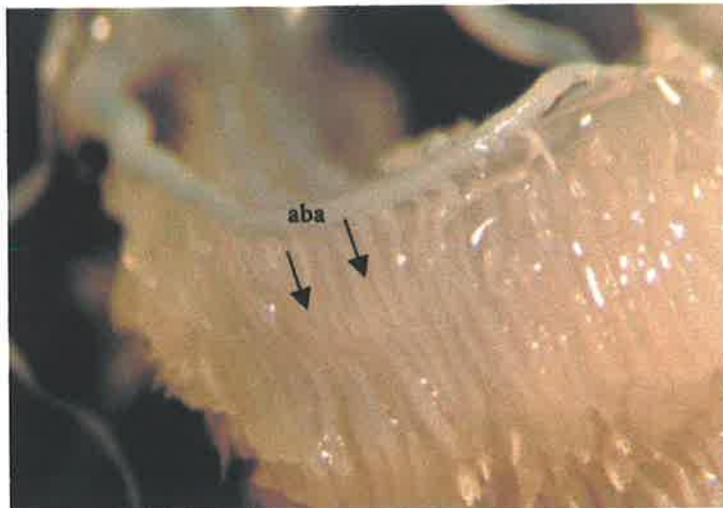


Figure 2.25 From each individual gill vessel, an afferent branchial artery (aba) entered each primary gill filament (see arrow direction). The gills demonstrated here are from a stage 11 embryo.

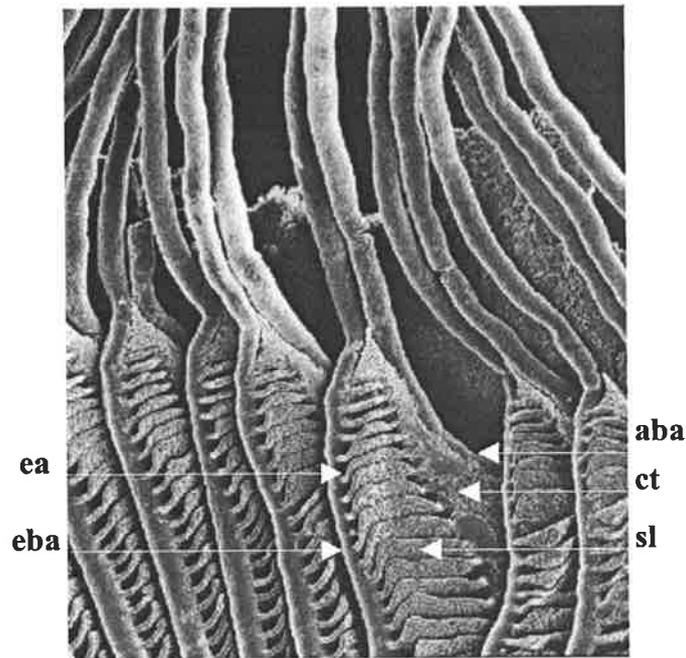


Figure 2.26 Blood flows from the afferent branchial arteries (aba) into the afferent arterioles (not visible), through the cavernous tissue (ct) and then through the secondary lamellae (sl). The now oxygenated blood is then transported through the efferent arterioles (ea) to the efferent branchial arteries (eba). n.b From a stage 11 embryo.

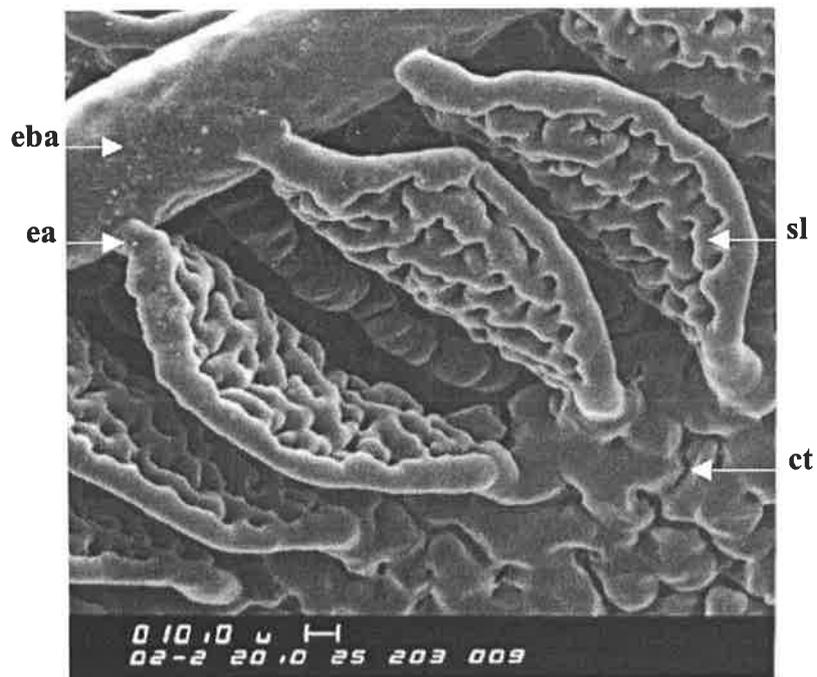


Figure 2.27 A close up of the cavernous tissue (ct), the secondary lamellae (sl), efferent arterioles (ea) and the efferent branchial artery (eba) in a stage 11 embryo.

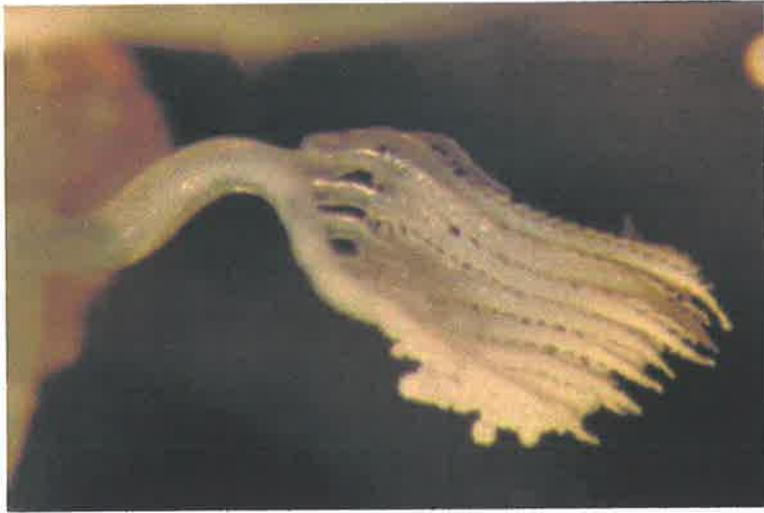


Figure 2.28 Blood vessels leading to and from the spiracle in the stage 11 embryo.

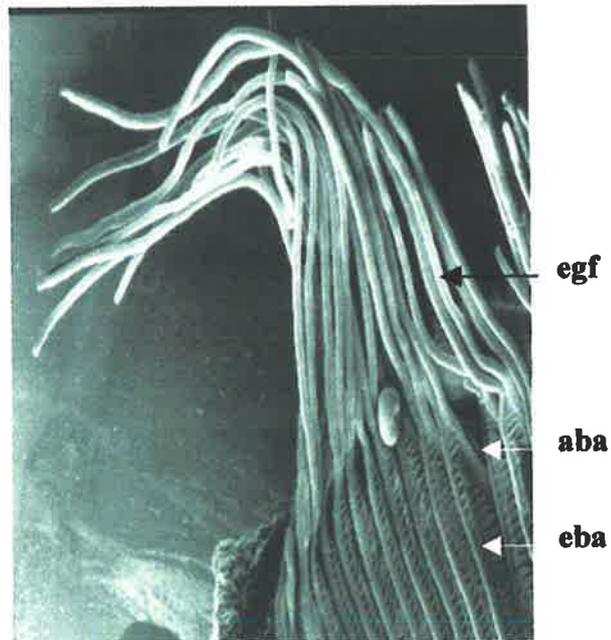


Figure 2.29 A scanning electron micrograph showing the relationship between internal (aba and eba) and external vasculature (egf) of the gills.

## 2.4 DISCUSSION

### 2.4.1 Staging procedure

There are many published studies describing different developmental stages in elasmobranchs, but most of these are inadequate as stand-alone documents in describing the entire elasmobranch development. Several of these contained gaps in the developmental stages and lacked time frames for the appearance of each morphological stage. Many of these were descriptions arising from the opportunistic collection of elasmobranch embryos from reproductive studies (Shann 1923, Baranes and Wendling 1981, Fujita 1981, Gilmore et al. 1983, Natanson and Cailliet 1986, Castro et al. 1988, Gilmore 1993 and Wenbin and Shuyuan 1993). Some concentrated on only the early or later stages of development (Clark 1926, Te Winkel 1950, Ballard et al. 1993), while Castro and Wourms (1993) emphasised stages pertinent to their study on placental development. Balfour (1878) used several different species and had no time frame for his staging, although the descriptions of the early stages were exceedingly detailed. While it is recognised that gaps in the descriptions are regrettable, they are often unavoidable because of difficulties in gaining specimens, particularly viviparous ones. However the detail described in the above studies allows for comparisons to be made between species and reproductive strategies.

The most complete staging description of oviparous elasmobranch development to date study has been the work of Dean in 1906 (Smith 1942), which documented the development of another Heterodontid species, *H. japonicus*. He detailed development from the early gastrula to hatching. While it did not attribute stages to embryos or have a definite time frame, the descriptions allowed a better understanding of *H. portusjacksoni* development. From the literature I have constructed a table which compares the relative appearance of common morphological or physiological features (as % of incubation) in several oviparous species (Table 2.6). I have included only those studies from which a time frame could be determined. As can be seen, many of the features appeared at similar time frames within the incubation. It is interesting to note that the external gill filaments appeared relatively earlier in development in *S. canicula* and remain longer in *S. retifer*, perhaps reflecting greater respiratory demands.

<b>Morphological / physiological feature</b>	<b><i>Heterodontus portusjacksoni</i> (this study)</b>	<b><i>Scyliorhinus retifer</i> (Castro et al. 1988)</b>	<b><i>Scyliorhinus canicula</i> (Ballard et al. 1993)</b>	<b><i>Raia brachyura</i> (Clark 1926)</b>
Cephalic flexure	10		10	
Gill arches	14	22	14	4
Heart activity	19		12	
Body movement	19		10	18
Opaque body	29	49		
Fin budding	29	31	29	34
External yolk vascular	29		23	
Yolk stalk reduced	29		15	
External gill filaments	31	34	16	34
Retinal pigment	33		26	
Mouth recognisable	36		25	
Dorsal spine	36		36	69
Claspers	38		36	53
Capsule opens	35-40	40-45	36	25-32
Body pigment	48		51	69
Resorption of external gills	48	70	51	62
Internalisation of yolk	48	76	51	
External yolk absorbed	71		77	
Incubation period (months)	10.5	8.4	6.4	6.6

**Table 2.6** The relative appearance of selected morphological and physiological features in the development of four oviparous elasmobranch embryos. The values represent the timing of the features as a percentage of the whole incubation.

Ideally, a complete staging procedure should include the very early developmental stages. However this assumes a knowledge of the date or time of fertilisation, a detail not always known. Therefore my stage 1 describes the morphology of development within the first month of existence external to the female. Examination of the ovaries for the presence of partially developed eggs would be necessary to be able to describe the very early stages. McLaughlin and O'Gower (1971) examined mature *H. portusjacksoni* females and suggested that eggs are shed fairly soon after fertilisation because of the relative lack of development of eggs within the uteri. The female lays up to 16 eggs in one breeding season lasting 2-3 months (McLaughlin and O'Gower 1971). Eggs are deposited in pairs at intervals of 1-2 weeks, not allowing time for substantial development to occur before laying. Production of egg capsules is even more rapid (1-4 days) in the skates, *Raja eglantaria* and *R. clavata* (Libby 1959, Libby and Gilbert 1960, Ellis and Shackley 1995), supporting the idea that little development occurs within the female. However, the first 4-5 stages of development described by Ballard et al. (1993) (pre-gastrula stage) in *S. canicula* occur within the uterus over a period of 3-5 days before the egg is released.

#### **2.4.2 Effect of temperature on development rate**

Development of ectothermic embryos occurs within a range of environmental temperatures bound by lower and upper vital limits, and within that range, development is directly influenced by the immediate temperature. Within the vital range of development, there may be an optimal temperature at which development rate is maximised, above and below which development is reduced. Alternatively, development may have a more linear relationship with temperature. Within the temperature constraints of this study, the latter appears to be the case for *H. portusjacksoni*. In the laboratory, under conditions of constant incubation temperature, development at 18°C, as defined as the appearance of particular morphological features, is approximately one month behind development at 20°C which occurs one month behind 22°C (Figure 2.24, p. 38). Some studies on teleosts have shown that the timing of significant morphological events related to feeding, such as hatching, the appearance of eye pigment and yolk sac absorption, were strongly dependent on temperature. The appearance of these events showed a curvilinear relationship to temperature in scad, *Trachurus trachurus* (Pipe and Walker 1987) and sardines, *Sardina pilchardus* (Miranda et al. 1990). The latter required 117h at 11°C to reach stage 10, while

reaching the same stage after only 39h at 20°C. This temperature dependency, although less marked, was repeated for other stages of development.

### **2.4.3 Albumen and osmo-regulation**

While the egg capsule is still sealed, the embryo is bathed in a protective albumen, provided by the mother at the time of laying. The albumen surrounding the embryo is thin while a thick gelatinous albumen extends from the plugs and lines the inner surface of the capsule. The functional significance of albumen has been suggested as being mechanical protection (Price and Daiber 1967). The albumen acts to cushion both the embryo and the yolk against external movements, which might otherwise disrupt the yolk membrane and cause death. In the early stages, the yolk membrane is extremely fragile and can break very easily (Smith 1942, Ballard 1973, Ballard et al. 1993, this study).

It is hypothesised that the albumen also acts as an osmoregulatory aid while the egg is closed (Smith 1936). Until the embryo develops a functional osmotic regulating mechanism, the albumen provides a constant ionic environment. This allows more energy to be spent on development and growth, rather than osmoregulation which can be energetically expensive (Bone and Marshall 1982). Smith (1936) suggested that this is achieved because the capsule is impermeable to urea (the major osmotic constituent of elasmobranch blood), thus maintaining the high osmotic pressures found in the albumen. In contrast, studies by Foulley and Mellinger (1980a) and Evans (1981) show that the egg capsules of the elasmobranchs *S. canicula* and *Raja binoculata* are highly permeable to urea. This would lead to diffusive loss of urea to the seawater and therefore dispute an osmoregulatory function. If this is the situation, osmo-regulation must develop early in the development of embryos. Evans and Oikari (1980) believe this is the situation in the viviparous shark *Squalus acanthias*, which appears to be able to osmoregulate while still enveloped in a thin membranous casing inside the uterus, and in the oviparous skate, *R. erinacae* (Kormanik 1993).

Since there is no evidence of urea in the albumen, it is likely that the barrier to urea loss resides in the vitelline membrane (Hornsey 1978, Foulley et al. 1981, Ballard et al. 1993) or in the gill epithelia (Kormanik 1993) instead of the outer casing. This would lead to little or no urea diffusing into the albumen or loss to the external medium. Urea would remain in the yolk and embryonic circulation where it functions to maintain

osmotic pressures. The study by Price and Daiber (1967) on several elasmobranch species reported that oviparous embryos must be supplied with "a diffusion-proof egg capsule" to enable the embryo to survive until its osmoregulatory systems function. They based this statement on high levels of urea found in the egg capsule fluid, but Smith (1936) stated that the urea is located in the yolk, and not in the surrounding albumen. This further supports a semi-permeable yolk sac membrane as the responsible agent for internal osmoregulation. Low urea levels in the albumen were also implied in this study for *H. portusjacksoni* eggs. The total osmotic pressure and chloride ion concentrations of albumen samples from *H. portusjacksoni* are comparable to those of *R. erinacae* (Kormanik 1993) and slightly hyper-osmotic to the surrounding seawater (Table 2.3, p. 22). This suggests that there is little or no urea in the albumen (assuming that NaCl makes up approximately 90% of the total osmotic concentration as it does in seawater (Potts and Parry 1964)). In contrast, the total osmotic and chloride concentrations of fresh yolk samples is 677 mOsmol/kg and 93 mmol/kg, respectively. This creates a very large gradient for chloride ions across the yolk sac membrane which is maintained through membrane impermeability (Gibbs-Donnan equilibrium) or active transport. The small percentage of the total osmotic concentration accounted for by chloride ions in the yolk (14%) suggests a large solute component of organic materials such as urea, proteins, lipids and carbohydrates. Because the yolk and therefore its components are in direct contact with the embryonic circulation, through the vitelline circulation, it is reasonable to assume osmotic regulation of the embryo takes place by this route. While it is probable that urea regulation occurs within the egg, it is the yolk membrane and gill epithelia that are responsible, not the surrounding albumen (Smith 1936, Read 1968, Shuttleworth 1988, Kormanik 1993), disputing an osmotic function for albumen. The yolk membrane is the main osmotic barrier in the eggs of the freshwater turtle, *Chelodina rugosa*, which lays its eggs underwater where they can undergo a period of developmental arrest for more than 12 weeks (Seymour et al. 1997).

#### **2.4.4 The rate of egg capsule mass loss**

The total mass of the egg capsule (plus mucous plugs) declines with time in the first 2 months (Figure 2.6, p. 24). The decline is rapid at first (0.52 g/d) and then slows down to a constant rate of loss (0.23 g/d). The progressive measurements of mass and capsule thickness indicate that the decline in mass is not a function of decreased capsule thickness (there was no significant change in thickness), but due to the mucous plugs

and the associated albumen dissolving. This explains the higher rates of loss at the beginning where there is still some thick albumen attached to the mucous plugs. After these dissolve, the rate of loss is relatively constant. In addition, observations on the half egg capsules in which the mucous plugs are visible, show the plugs decreasing in overall surface area, until they disappear. The study by Mellinger (1983) supports the loss in egg capsule mass as albumen losses, rather than egg capsule deterioration.

#### **2.4.5 Opening of the egg capsule**

Mellinger (1983) generalised that the albumen dissolves, and the egg capsule opens, at approximately midway through incubation in chondrichthyan fishes. This study and reports from the literature suggest this to be a slight overestimate. The egg capsules of the ray, *Raia brachyura*, and skates, *Raja eglanteria*, and *R. erinacea* open at 25-32% of incubation (Clark 1926, Libby 1959, Libby and Gilbert 1960, Long and Koob 1997). In the cat shark, *Scyliorhinus retifer*, the capsule opens at 45-50% of incubation (Castro et al. 1988), while opening occurs between 36-45% of incubation in the Lesser Spotted dogfish, *S. canicula* (Collenot 1966, Ballard et al. 1993).

The mucous plugs dissolve at about 35-40% of incubation in *H. portusjacksoni*. The dissolution of the plugs and vigorous embryonic movement allows seawater to circulate through the capsule. Any physiological restraints that may have existed, in particular oxygen, are now removed and development is limited only by the capsule volume and environmental variables such as temperature. It is likely that the capsule is diffusion limiting to the growing embryo (Chapter 5, section 5.4.2, p. 161) and therefore the opening of the egg capsule to seawater increases the capacity for growth supported by aerobic metabolism.

##### **2.4.5.1 The role of embryo on egg capsule opening**

The average timing of opening of an *H. portusjacksoni* egg capsule at all temperatures was late January. The opening date was unaffected by the removal of the embryo, producing similar opening dates in control egg capsules from which the embryo was removed. This leads to the rejection of the hypothesis that egg capsule opening is controlled by the embryo; rather it is a predetermined event. There must be a change in the thick albumen surrounding the mucous plugs that occurs over time, however the biochemical mechanism responsible is unknown. The ability to anticipate the capsule opening date has implications which allow the ageing the early embryo. Eggs are laid

over two to three months period (late August to mid November), therefore it is not surprising to see a similar range for opening dates (late December to late February). This represents an interval between laying and opening of approximately 120 days for each egg. Using the 120 day period of egg capsule closure, the age of a sacrificed embryo from a closed capsule could be estimated if the mucous plugs were left to continue incubation after the embryo has been removed.

The passive opening of *H. portusjacksoni* capsules was contrasted by the only other report on elasmobranch development that described opening actively initiated by the embryo (Ballard et al. 1993). A hatching gland located on the rostrum was responsible for the opening of the egg capsule of the Lesser Spotted dogfish, *S. canicula*. Secretions from the hatching gland digested the albumen content of the egg capsule (including the plug), allowing seawater to enter the capsule. Having digested the albumen, the gland was presumably reabsorbed before hatching occurs. Capsule opening may still have occurred in the absence of the dogfish embryo, but this was not examined. No hatching gland was observed in *H. portusjacksoni*.

#### **2.4.6 Yolk sac vascularisation and yolk stalk**

The yolk is surrounded by a yolk membrane that is made up of five layers (Hamlett and Wourms 1984). The innermost layer is the yolk syncytium which functions to remove nutrients from the yolk by endocytosis. The next layer is the endoderm which acts as a mediator of the yolk nutrients. The middle layer is the vitelline circulation which transfers the nutrients from the yolk to the embryo. This layer is protected by a mesoderm and ectoderm on the outer surface.

There are two functions attributed to the yolk sac membrane; nutrition and respiration. Nutrients are broken down by enzyme reaction in the yolk itself (Jollie and Jollie 1967) and by reactions in the syncytium (Hamlett 1989b). They are transferred from the external yolk into the vitelline circulation. In avian yolk membranes, it is the endodermal cells located next to the yolk which function to mobilise and process yolk nutrients. These cells control the release of nutrients into the vitelline circulation which delivers the nutrients to the embryo's tissues (Richards 1991). In addition to nutrients, the yolk sac membrane is thought to absorb water and other substances from the fluid within the egg capsule (Te Winkel 1963a, Wourms 1981).

The respiratory role of the yolk sac membrane has been suggested by many authors (Te Winkel 1963a, Jollie and Jollie 1967, Hamor and Garside 1977, Baranes and Wendling 1981, Hamlett and Wourms 1984). The necessary vascularisation of the membrane begins early in the developmental history of *H. portusjacksoni*. By stage 5, the vitelline circulation has begun and by stage 6 it is quite extensive. Vascularisation has the added function of strengthening the yolk membrane against being ruptured. Ballard et al. (1993) found that it was difficult to remove an embryo prior to membrane vascularisation without destroying the continuity of the yolk membrane and causing death. Vascularisation of the external yolk sac remains extensive while the yolk is present, a feature also observed in the Japanese species, *H. japonicus* (Smith 1942). Comparative observations of yolk sac membrane morphology in other elasmobranch species are not possible because the descriptions of embryonic development concentrate on morphology of the embryo and ignore the yolk sac.

Jollie and Jollie (1967) investigated the differential functions of the yolk sac in the ovoviviparous Spiny dogfish, *Squalus acanthias*, using microscopic examinations at various periods of the long gestation (up to 18 months). They found that the yolk sac has two barriers through which nutrients and gases are exchanged, termed the yolk-blood barrier and the respiratory barrier. The transport of the nutrients and respiratory gases are separate and achieved by several differentiated cell layers. Similarities can be drawn to oviparous embryos.

The function of the yolk sac varies with the developmental stage of the embryo (Jollie and Jollie 1967). In the early developmental stages of *H. portusjacksoni* and *S. acanthias*, the embryo is just beginning to develop and the yolk sac serves both a nutritive and respiratory function. The yolk sac membrane has a vast network of vessels which assist in these functions. The major artery (vitelline) extends from the embryo into the yolk sac membrane. Associated with the artery are many hundreds of fine vessels branching from the main artery, most often at right angles. These can be seen in Figures 2.13, (p. 28) and 2.20a (p. 32). At the venous side of the yolk, many contributories form the umbilical vein that empties into branchial area and then to the systemic system. The respiratory function of the yolk sac wanes as the embryo develops external gill filaments.

As development proceeds in the *H. portusjacksoni* embryo, the yolk-stalk connection between the external yolk and embryo decreases from being extensive (stage 4 embryos are connected over one third of their body length - Figure 2.11, p. 27) to a small discreet yolk-stalk in the stage 6 embryo (Figure 2.13, p. 28). The yolk-stalk has several vessels through which blood and yolk are transported (Figure 2.14, p. 29). By late-gestation, the yolk sac in *Squalus* embryos is not responsible for either nutrient or gaseous exchange (Jollie and Jollie 1967). The nutrients contained within the yolk sac are transported directly to the spiral intestine through the central ductus vitello-intestinalis of the yolk stalk. It is likely that as the embryo grows, the effective surface area for absorption of yolk nutrients by the membrane decreases and may become limiting in terms of nutrient uptake. Direct delivery to the intestine through the ductus vitello-intestinalis overcomes this limitation.

The alterations in yolk sac function in *S. acanthias* are correlated to structural changes in the cell layers of the separate barriers. Keratinisation of the ectoderm, thickening of the underlying layers and production of additional smooth muscle layers above the vitelline circulation lead to an increased respiratory barrier, ultimately resulting in complete blockage to all respiratory gases. Nutrient transfer ceases in the second year of development in *S. acanthias* as the cellular layers of the yolk-blood barrier change (Jollie and Jollie 1967).

#### **2.4.7 Internal yolk**

During stage 12, an internal yolk sac is formed in the abdominal cavity of developing embryos. Yolk from the external sac leads directly to the internal yolk sac (through the ductus vitello-intestinalis) which in turn leads into the spiral gut of the embryo. Cilia along the walls of the stalk are responsible for the transfer of the yolk (Te Winkel 1963b, Baranes and Wendling 1981, Hamlett 1989b). Thus yolk is transferred from the external sac and stored in the internal sac. The internal sac is not vascularised, indicating storage function only.

The transfer of yolk from an external sac to an internal one is common among elasmobranch embryos (Gilmore et al. 1983, Wolfson 1983, Hamlett and Wourms 1984, Natanson and Cailliet 1986, Castro et al. 1988, Capape et al. 1990, Yano 1992, Ballard et al. 1993). Internalisation of yolk may afford greater protection for the yolk in the event of premature hatching. *H. portusjacksoni* begins to internalise yolk at stage 12, 4

to 5 months prior to hatch (48% of incubation). By stage 14, the external yolk is absent, and the internal yolk sac is at maximum mass (Chapter 3, Figure 3.5, p. 74). Over the next three months, the internal yolk stores are consumed. Hatchlings have a small (<1% of body mass) internal yolk reserve so they must feed soon after hatching. Comparative data for internalisation of yolk are poor for elasmobranchs. Castro et al. (1988) report initiation of internalisation of yolk some time after 75% of incubation in *S. retifer*. Data from other elasmobranch studies show the presence of internal yolk but fail to include details regarding its formation and duration (Gilmore et al. 1983, Wolfson 1983, Natanson and Cailliet 1986). The study by Clark (1926) was based on external morphology only and fails to discuss internal yolk. The embryos in reptilian (Ricklefs and Cullen 1973, Stewart and Castillo 1984, Ewert 1985, Deeming and Ferguson 1989, Whitehead et al. 92) and avian (Vleck et al. 1980, Bucher and Bartholomew 1984) eggs also internalise yolk late in the development process. In the majority of the resulting hatchlings, there is still an internal reserve which provides maintenance energy for the hatchling during the early days of self feeding (Stewart and Castillo 1984, Allsteadt and Lang 1995). This is supported by the fact that precocial chick hatchlings, which must feed themselves, have a greater internal supply than altricial hatchlings, which are fed by the parents for some time after hatch (Vleck et al. 1980). Internalisation of yolk stores for the reptilian and avian embryos is a condition of survival once hatched.

#### **2.4.8 Gill vasculature**

The systemic circulation of both elasmobranchs and teleost fish consists of afferent and efferent vessels which give rise to afferent and efferent arterioles just before and after the secondary lamellae (Randall 1982a, Nilsson 1986). The afferent vessels enter the secondary lamellae with deoxygenated blood and the efferent vessels leave with oxygenated blood which enters the dorsal aorta and systemic flow (Figure 2.26, p. 40). The elasmobranchs have an additional circulatory bed which lies between the afferent circulation and the lamellae called the 'cavernous tissue' (Figure 2.27, p. 40) (Randall 1982, Laurent 1989). This tissue may function to support the primary filaments (Wright 1973, Cooke 1980, De Vries and De Jagger 1984). The cavernous tissue is absent in teleost circulation, because of the presence of skeletal support (Randall 1982). It may also function in the phagocytic destruction of red blood cells and other particles (Wright 1973, Hunt and Rowley 1986), or as a pulse-smoothing capacitance vessel (Wright 1973).

While the association between the external and internal gill structures is known (Kryvi 1976, Hamlett et al. 1985), it has not been examined using vascular casting techniques. My investigation supported these earlier studies in demonstrating that the external gill filaments are capillary loops that extend from the afferent and efferent arterioles of the primary filament (Figure 2.29, p.41).

#### 2.4.8.1 External gills

Many aquatic embryos develop external gill filaments during incubation, for example, amphibians (Rugh 1951, Salthe and Mecham 1974), teleosts (Ballard 1973, Blaxter 1988), and elasmobranchs (Balfour 1878, Clark 1926, Libby 1959, Te Winkel 1963a, Jollie and Jollie 1967, Kryvi 1976, Baranes and Wendling 1981, Fujita 1981, Gilmore et al. 1983, Hamlett and Wourms 1984, Natanson and Cailliet 1986, Castro et al. 1988, Pelster and Bemis 1991, 1992, Wenbin and Shuyuan 1993, this study). External gill filaments contribute significantly to the oxygen uptake in the early skate embryo, *R. erinacae*, supplying up to 50% of the total oxygen requirements (Pelster and Bemis 1992). External gills are thin-walled and highly vascular, fundamental requirements for effective respiratory exchange. Their presence in the clawed frog, *Xenopus laevis*, contributed significantly to the aerobic respiration, particularly when exposed to hypoxia (Hastings and Burggren 1995).

External gill filaments are formed as extensions to the gill arches. The gill arches appear within the first 22% of incubation of elasmobranch development (Table 2.6). *H. portusjacksoni* embryos showed the first signs of gill arches during Stage 4, at 14% of incubation, and they first appear in *R. brachyura*, *S. canicula* and *S. retifer* between 4-22% of incubation (Clark 1926, Castro et al. 1988, Ballard et al. 1993). The filaments appear early in elasmobranch development and are lost well before hatching or birth. This study shows the first appearance of external filaments in stage 7 embryos (observed as small tufts) at 25-30% of incubation (Figure 2.15, p. 29). Filaments typically appear in a rostral to caudal sequence as observed in the skate, *Raja erinacae* (Pelster and Bemis 1992) and Lesser Spotted dogfish, *S. canicula*, (Ballard et al. 1993) with the spiracular filaments appearing last (Castro and Wourms 1993). Other studies report similar timing of filament appearance in oviparous elasmobranch development (Clark 1926, Pelster and Bemis 1991,1992, Castro et al. 1988). In the ovoviviparous Pacific angel shark, *S. californica*, they appear much earlier (10%) in development

(Natanson and Cailliet 1986). Dean (in Smith 1942) recorded external filaments in very early embryos of *H. japonicus*. Unfortunately, there was no indication of the size of the embryo when this stage was reached, or of any time frame from which the stage, relative to the total incubation period, could be deduced.

As the embryo increases its tissue mass and the oxygen demands increase, the embryo needs to increase the amount of oxygen made available to the cells of the body. The development of external gill filaments increases the embryonic surface area and enhances respiratory gas exchange. In addition, the gill epithelia of elasmobranchs is believed to have primary importance in the retention of urea (Smith 1936, Read 1968, Shuttleworth 1988, Kormanik 1993). Therefore, until the embryo develops functional osmoregulation, the appearance of osmotic aids in the form of external gill filaments would be an advantage.

Once the egg capsule is open to seawater, movement of the embryo draws seawater into the capsule and the embryo can take up oxygen as required. At this time, the external gills are not necessary to the same degree as prior to opening, and they are resorbed back into the internal gills from where they originated. The osmotic function of the external gills is also reduced at egg capsule opening. By the time the embryo is exposed to seawater, it must be able to osmoregulate. Maximum filament development occurs just after capsule opening, at stage 11, and resorption takes place within one month at stage 12. During the resorption of the external gills, active internal gill movements occur, further reducing the need for external gill filaments as respiratory organs. Pelster and Bemis (1992) report that the internal gills become functional as the external gill filaments fail to provide enough oxygen to the developing skate embryos, *R. erinaceae*.

The external gill filaments of embryonic *H. portusjacksoni* are completely resorbed at 45-50% of the incubation, soon after the capsule opens. This is observed in many oviparous elasmobranchs. The resorption of filaments occurs at 62% of the incubation in *R. brachyura* (Clark 1926), 70% in *S. retifer* (Castro et al. 1988) and at 50% in *R. erinacea* (Pelster and Bemis 1991). In live bearing species, the filaments are lost earlier in the gestation. The ovoviviparous Pacific Angel shark, *S. californica*, has lost the external filaments at approximately 28% into the gestation (Natanson and Cailliet 1986). The external filaments are gone approximately 20% into the gestation when the viviparous *Rhizoprionodon terraenovae* embryo implants into the uterine wall (Castro

and Wourms 1993). The loss of external filaments coincides with the development of the placenta and with the appearance of appendiculae; highly branched, filamentous structures that cover the umbilical cord. Gas exchange functions are hypothesised to be taken over by the placenta or the appendiculae, thus eliminating the need for external gill filaments.

A nutritive function has been hypothesised for external gill filaments in elasmobranch embryos (Shann 1923, Gilmore et al. 1983, Hamlett et al. 1985). The epithelial cells of the external filaments contain Golgi bodies, fibrils, mitochondria and phagocytic vesicles, supporting a nutritive function (Kryvi 1976). Secretions from the uterine wall may be absorbed by external gill filaments of the porbeagle (*Lamna cornubica*) and sand tiger sharks (*Odontaspis taurus*) (Shann 1923, Gilmore et al. 1983). These elasmobranch species are viviparous and the developing embryos are found free-swimming within the uterine cavity for the majority of gestation. Nutrition is provided initially by an attached external yolk sac, and later by uterine wall secretions which presumably are taken in through the mouth and gills. Hamlett et al. (1985a) have produced some evidence for a nutritional function of external gill filaments in the viviparous embryonic shark, *R. terraenovae*. They showed that the squamous epithelial cells lining the filaments absorb horseradish peroxidase, a marker for protein absorption, under *in vitro* conditions. However, such a nutritional function for external gill filaments is less likely in oviparous forms where the surrounding medium (albumen) is not continuously enriched with nutrients by the mother, and the embryo depends on the yolk sac for its nutrients throughout incubation.

#### **2.4.9 Heart activity**

During the development of the gill arches, from which the gill filaments later emerge, the heart region becomes active. Cardiac development usually precedes the development of other organ systems because of the necessity of delivering nutrients and facilitating gas exchange to them (Pelster and Bemis 1991, Rombough 1997). The development of the heart in the Little Skate, *R. erinacea*, is extremely rapid, such that within 2 weeks, the heart has most of the features of an adult heart (Pelster and Bemis 1991). The elasmobranch heart consists of four chambers, the sinus venosus, atrium, ventricle and conus arteriosus. In addition, valves are found between the chambers in order to control the flow and direction of blood flow (Hamlett et al. 1996b), although

these may not be evident until later (Pelster and Bemis 1991). Active pumping of the heart, observed as early as 15-20% of incubation in the stage 5 *H. portusjacksoni* embryo (this study) and 16% in the Little Skate, *R. erinacea* (Pelster and Bemis 1991), enhances circulation of blood through the body and yolk.

#### **2.4.10 Development of fins**

The development of fins from the fin fold that surrounds the elasmobranch embryo can greatly increase the effective surface area of an embryo. This is particularly important as the skin is a major respiratory organ in early embryonic forms (Hamor and Garside 1977, Hamlett and Wourms 1984, Stewart and Castillo 1984, Rombough and Ure 1991, Castro and Wourms 1993). Fin budding occurs close to the time at which external gill filaments first appear at a time when the embryo needs to increase its surface area for cutaneous respiration. In this study, fin budding occurred in stage 6 embryos or at 25% of incubation. In *R. brachyura* and *S. retifer*, it occurs between 30-35% of incubation (Clark 1926, Castro et al. 1988).

#### **2.4.11 Hatching**

Hatching in fish can be stimulated by several intrinsic and extrinsic factors such as temperature, oxygen availability, light and water turbulence (Blaxter 1969, Yamagami 1988, Helvik and Walther 1993). Hatching occurs earlier at higher temperatures where oxygen demands are greater and yolk reserves are utilised more quickly (Herzig and Winkler 1976, Pipe and Walker 1987, Miranda et al. 1990, Arul 1991).

Hypoxia is known to induce hatching in several aquatic groups. Low oxygen retards growth, but also stimulates early hatching of less advanced embryos (Hamor and Garside 1977) because of increased mobility (Keckeis et al. 1996). Metabolism of many teleost species was reported to increase immediately at hatching, suggesting that the capsule had become oxygen limiting, thus forcing the embryo to hatch (Holliday et al. 1964, Eldridge et al. 1977, Davenport and Lönning 1980, Davenport 1983, Rombough 1988b). Alderdice et al. (1958) calculated critical oxygen tensions in the eggs of Pacific salmon, *Oncorhynchus keta*, during development. They found that hatching in advanced embryos occurred in response to low levels of oxygen. In contrast, hatching was inhibited by hypoxia and turbulent water in halibut, *Hippoglossus hippoglossus*, while darkness stimulated the hatching process (Helvik and Walther 1993). Removal from turbulent water into calm conditions was enough to

induce hatching. They also found that hypoxia prevented hatching because there was not enough oxygen present for the actual hatching event.

Increasing hypoxia due to diffusing limitations of the capsule can cause amphibian embryos to hatch (Seymour 1994). Hatching in marbled salamanders, *Ambystoma opacum*, occurs when environmental PO<sub>2</sub> falls below 86 Torr (Petranka et al. 1982) as a result of flooding. Similarly, flooding produces a boundary layer around the capsule and reduces the PO<sub>2</sub> of the eggs of the frog *Pseudophryne bibronii* and stimulates hatching (Bradford and Seymour 1988). Low oxygen levels in the foam nest of the African frog, *Chiromantis xerampelina*, have been hypothesised to cause larvae to leave the nest (Seymour and Loveridge 1994).

It is also likely that food (or energy) availability may influence hatching in *H. portusjacksoni*. Late in stage 14, the *H. portusjacksoni* embryo appears as a miniature adult. The external yolk is absent and there is a large internal yolk (approximately 120g). Externally, the embryo appears developmentally complete, and if it hatched prematurely at this stage, the embryo could conservatively survive on the internal reserve for approximately 125-150 days if there was minimal growth (based on metabolic demands of a hatchling). However, under constant laboratory conditions of temperature and light, the embryo does not hatch at stage 14. It remains in the egg capsule for a further three months until the internal reserves are depleted. The delay to hatching may be that there are developmental processes not yet complete, which require more maturation time. The internal yolk reserves found in hatchlings amount to less than 1g. This reserve is equivalent to a maximum of 11 kJ of energy (calculated using Tables 3.1, p. 73, 4.1, p. 103 and 4.3, p. 105) which would sustain the embryo for approximately 9 days, hence the embryo must hatch in order to find an exogenous energy source. This suggests that energy availability is a strong influence on hatching under laboratory conditions. This contrasts the situation in many teleost species which hatch as pro-larvae with a substantial yolk attached (Gruber and Weiser 1983, Quantz 1985, Rombough 1988b).

The amount of endogenous energy sources in other oviparous elasmobranch hatchlings has not been documented. However, Natanson and Cailliet (1986) found no evidence of yolk (external or internal) in any pups of the ovoviviparous Pacific Angel shark, *S. californica*, hypothesising that the complete exhaustion of yolk supplies is timed with

parturition. Other studies have implied internal yolk stores in near-term viviparous embryos. Yano (1992) describes near-term embryos of the black dogfish, *Centroscyllium fabricii*, with substantial internal yolk stores (1.2-4.1 g), however the timing between near-term and birth is not reported, so a significant amount of this may be used up before birth. A single whale shark, *Rhincodon typus*, pup (one of approximately 300) removed from a deceased female did not begin external feeding until 17 days after birth, suggesting significant internal nutrient stores (Chang et al. 1997). However it is likely that the birth was premature due to the death of the mother and the pup may not have hatched until much later when stores were markedly less.

The stimuli for hatching in other groups of animals is not always known. In crocodylian embryos, it may be influenced by mechanical stimuli and vocalisations. Young crocodiles are known to vocalise as they hatch (Spellerberg 1982), signalling the female adult to excavate the nest and aid in the escape from the nest environment (Webb and Manolis 1989). In addition, increasing levels of waste material, decreasing egg water content and inadequacy of gas exchange to provide enough oxygen to maintain metabolism at the current rate may affect the hatching process in reptiles (Ferguson 1985). Evidence from this study also suggests that water quality may stimulate premature hatching. On two occasions, the death of one embryo fouled the water and induced the other embryos in the aquarium to hatch, sometimes 2-3 months early. Presumably the embryo hatches to remove itself from a poor water environment into a better one.

## 2.5 SUMMARY

Among the oviparous elasmobranchs, *H. portusjacksoni* lays one of the largest eggs, weighing an average of 155.5 g and containing a single embryo. The egg is surrounded by a thick conical capsule which has two respiratory slits located at either end. The slits are sealed by a plug of mucous for the first 40% of incubation (equivalent to four months). The egg contains a large yolk, immediately surrounded by a yolk-sac membrane, which sits in a bath of albumen. The albumen is slightly hyper-osmotic to seawater in terms of total osmotic pressure and chloride concentrations. In contrast, there are large osmotic gradients between yolk and surrounding albumen, with yolk being about half the total osmotic concentration of albumen. In terms of chloride ions, yolk has only 16% of the levels found in albumen. The albumen is largely salt water in composition, while the yolk primarily contains organic solutes. Large molecules and compounds such as urea, proteins and yolk nutrients are entrapped within the yolk by a relatively impermeable yolk membrane, which provides the embryo with osmotic protection until it can osmo-regulate for itself. As development proceeds, the plugs dissolve and the capsule becomes open to seawater. The opening of the capsule is a temperature-independent, time controlled event, not initiated by the embryo.

A staging table consisting of 15 stages is proposed for *H. portusjacksoni* development from immediately after laying to hatching. Where possible, functional significance of morphological features is examined. In sealed eggs, early embryonic respiration depends greatly on cutaneous exchange. Increases in effective surface area are achieved by the vascularisation of the yolk and embryo and production of fins. In addition, the external gill filaments are an important morphological and physiological feature during the first four months of incubation as shown by micro vascular casting techniques. They appear early in development (stage 7) as tufts (small extensions of the efferent and afferent branchial arteries), and rapidly develop to full length in the stage 11 embryo, just after the egg capsule opens. These respiratory aids may become limiting as the embryo grows, thus prompting the dissolution of the mucous plugs and the opening of the capsule to seawater. Embryonic movements then draw water through the capsule. Coinciding with the opening of the capsule is the reabsorption of the external filaments back into the internal gill structure and the initiation of internal gill function. Within one month of capsule opening, the filaments are resorbed back into the gill structure.

The yolk-sac functions initially as both a respiratory and nutritional aid. The vitelline circulation transports both respiratory gases and yolk nutrients to the embryo. Later, limitations brought about by increasing embryo requirements, lead to other organs (such as external filaments) taking over respiratory function, while yolk nutrients are now transported through the yolk-stalk connection via the ductus vitello-intestinalis. Approximately five months into incubation, the external yolk has been used directly by the embryo for growth and development, and internalised as an internal yolk-sac (stage 12). Once the external yolk is gone, the internal yolk stores are utilised. Hatching occurs after 10-11 months when internal yolk stores are all but gone.

Comparable data from other oviparous species are poor, but it is apparent that while some morphological features are conservative in their timing (external gill filaments and fin budding), others (resorption of external gill filaments) can vary considerably. The differences are even more obvious between the different reproductive modes found within the elasmobranchs. These differences decrease the overall value of the staging technique for inter-specific development, particularly between reproductive strategies. However it is a useful tool among oviparous forms.

The effect of temperature on the appearance of developmental features showed a general linear trend. An increase in incubation temperature by 2°C results in stages appearing earlier by approximately one month, whereas a 2°C decrease in temperature slowed the developmental process by approximately one month.

## Chapter 3 GROWTH

### 3.1 INTRODUCTION

Oviparous elasmobranch embryos operate as semi-closed systems where the young develop within the protective environment of an egg capsule. The embryo is provided with all its organic requirements for growth by a large yolk mass (Hamlett 1989b). The only requirement of the environment is an appropriate temperature and facility for exchanging respiratory gases and eliminating waste products. The yolk is taken up by the developing embryo and used to build tissue mass.

Like other developing vertebrate eggs, fish eggs are generally located in a permanent position and must be tolerant of environmental changes in order to survive and develop successfully (Wang et al. 1987, Heming and Buddington 1988). The ability of fish to tolerate change is the basis of many investigations because fish are found in a wide variety of ecological niches and are exposed to variations in environmental temperatures (Alderdice and Forrester 1971, Morgan 1974, Johns and Howell 1980, Beacham and Murray 1990). The direct effect of temperature on growth, metabolism and development is often hidden behind the complex interaction of temperature, salinity, oxygen, light and food conditions (Herzig and Winkler 1986). Growth rates in both embryonic and adult fish are affected by salinity (Alderdice and Forrester 1971, Hettler 1976, Cheng-Sheng and Menu 1981, Lang et al. 1994). It has also been shown that the availability of oxygen has profound effects on metabolic rate and hence growth (Garside 1959, 1966, Hamor and Garside 1977, McCutcheon et al. 1982, Walsh and Lund 1989, Diez and Davenport 1990). The effect of temperature on growth of embryonic fish has been widely investigated. Increased temperatures decrease the incubation time (Rajagopal 1979, Herzig and Winkler 1986, Pipe and Walker 1987, Miranda et al. 1990, Pepin 1991), thus producing viable larvae more quickly, a desired result for hatchery economics.

The duration of incubation in oviparous embryos may also be influenced by egg size (Rahn and Ar 1974, Eldridge et al. 1982). Larger eggs tend to produce larger offspring which generally take longer to develop. The possibility of developmental arrest lengthens the period of incubation in reptilian embryos (Hubert 1985). Similarly, any slowed growth phases caused by environmental factors (such as a reduction in ambient temperature) result in a longer overall incubation period. Geographic location may

directly affect environmental variables such as temperature and oxygen availability, thus indirectly affecting the incubation period (Ewert 1985, De Marco 1990).

### 3.1.1 Allometry of growth

Growth can be described in a number of ways: with allometry, comparing one variable with another, and by examining the pattern and rate of growth over time. Allometry is the mathematical description of relations between an animal's body dimensions. Body mass is the primary allometric variable used but any morphological feature can be used. Allometry is important in examining the dynamics of growth, how one part of the animal is growing relative to another. The allometric relationship between mass and total length demonstrates the nature of change of body shape during development. The allometric relationship between mass and length can be represented by the power curve equation :

$$M = aL^b$$

where M is body mass, L is length, and 'a' (=elevation) and 'b' (=power of exponent) are parameters estimated by linear regression based on logarithms:

$$\log L = \log a + b \log M$$

If an animal is growing isometrically, that is, the length and mass are increasing at the same rate, then as the length doubles, the mass increases in relation to its volume by 2<sup>3</sup> or 8 fold (King 1995) and b is equal to 3. Allometric relations can be produced between any morphological variable and they can then be useful in estimating unknown variables from known ones. This is particularly useful for embryonic forms where some dimensions (such as mass) are difficult to measure accurately, or in estimating the theoretical mass of egg components in a fresh egg that is allowed to continue development. For example, the estimation of the wet mass of *H. portusjacksoni* embryos younger than stage 10 from their known length, and the calculation of the Gross Efficiency of Growth (**GEG**) (see later) from known initial egg mass and hatchling mass. The initial yolk mass can be estimated from the allometric relationship between the initial egg mass and yolk mass.

### 3.1.2 Patterns of growth

There are various patterns of embryonic growth observed among the different vertebrate groups. Growth can be sigmoidal as in most precocial avian and some reptilian

embryos (Ricklefs 1968, Vleck et al. 1980, Ackerman 1981b, Ewert 1985, Miller 1985, De Marco 1990, Whitehead et al. 1992), or exponential as in the squamates and crocodylians, altricial bird and mammalian embryos (Robertson 1923, Clark 1953, Dmi'el 1970, Ricklefs and Cullen 1973, Vleck et al. 1980, Deeming and Ferguson 1989). Sigmoidal growth is typified by an initial slow growth phase, followed by a rapid expansion phase, and finally by a slow plateau phase nearing hatching or birth. Sigmoidal growth can be described by three equations, the Logistic, Gompertz and von Bertalanffy equations. These equations differ according to the point of inflection (that is, initiation of the last phase), thus producing variable plateau phase lengths (Starck and Ricklefs 1998). The exponential growth pattern differs from the sigmoidal growth pattern in that there is no slowing of growth near the end of incubation. Exponential data are log transformed and analysed with linear regression statistics. The logistic growth equation proved to be the most appropriate for 77% of avian species examined by Ricklefs (1968). Precocial bird species are generally represented by the Gompertz equation, reflecting an earlier inflection point (Ricklefs 1973). However Whitehead et al. (1990) found that the logistic model was more appropriate for the precocial Magpie Geese hatchlings. De Marco (1990) employed the logistic equation in four species of lizards. Laird (1966) used a growth equation derived earlier and based on the Gompertz model (Laird et al. 1965), to compare mammalian and avian embryonic growth. Growth equations allow inter- and intra-specific comparisons to be made and are useful tools in determining the effect of specific variables, such as temperature, on growth.

The tremendous variety in the patterns of growth and development has prompted extensive research among avian (Carey et al. 1980, Vleck et al. 1984, Vleck and Vleck 1987, Starck and Ricklefs 1998) and reptilian embryos (Ricklefs and Cullen 1973, Ackerman 1981b, Thompson 1983, Whitehead 1987, Whitehead et al. 1992). Teleost fish have also received a great deal of attention, largely due to the growing importance of fish hatcheries (Marr 1966, Davenport and Lönning 1980, Johns and Howell 1980, Heming 1982, Houde and Schekter 1983, Escaffre and Bergot 1984, Alderdice 1985, Rombough 1988a, Finn et al. 1991). Unfortunately these studies have inevitably employed lethal examination of embryos and egg contents in order to understand the physiology of development and growth. In contrast, oviparous elasmobranch embryos, such as *H. portusjacksoni*, offer great advantages over these studies because individual growth and development can be examined sequentially in individuals. Once the egg

capsule is open, the embryo can be removed for periodic examination and then replaced so that it can continue normal development. The technique of removal of embryos from their egg capsules allows the progressive monitoring of growth and impact of environmental variables on embryos. The ability to monitor individual growth throughout the incubation period has tremendous advantages because the elements of inherent variability, created by individual egg and embryonic masses, are removed or at least markedly reduced.

### **3.1.3 Growth rate and the effect of temperature**

Under natural incubating conditions, where the temperature fluctuates, developmental processes are not under constant control. This is particularly true for aquatic animals in which development may occur over several seasons. During winter, when the temperature is lower, growth is slow compared to summer when growth is accelerated. Because of this, direct comparisons of natural incubations with laboratory controlled incubations are limited. It has also been shown that development of some squamate reptiles under constant temperature occurs at a greater rate than under naturally varying temperatures (Hubert 1985). For example, development of the lizard, *Lacerta vivipara*, proceeds twice as fast at a constant 27°C than in nature, with all developmental stages being normal. Notwithstanding, the underlying developmental processes can be investigated under conditions of constant temperature. The effect of different factors on growth can really only be assessed directly by controlling all other factors which will exert an influence. In this study, the variables of salinity and photoperiod were kept constant and growth was assessed at five environmental temperatures. The pattern of embryonic growth was monitored, using both live and dead material, to produce growth equations for this species and to establish allometric relationships. In addition, the effects of temperature and initial egg mass on hatchling mass and incubation period were investigated.

### **3.1.4 Gross efficiency of growth (GEG)**

Reduced incubation periods, induced by increased temperature regimes, may affect the embryo by altering the efficiency with which the embryo transforms the available nutrient source. This efficiency can be measured as a function of increased embryonic tissue mass as a result of decreased yolk mass. Marr (1966) used dry tissue increments of embryonic salmon to determine what Needham (1964) had termed the Gross

efficiency of growth (**GEG**). This is defined as the ratio of the increase in dry weight of the yolk-free embryo to the decrease in dry weight of the initial yolk, multiplied by 100. The term "gross" refers to the fact that the conversion efficiency can never be 100%, because some of the yolk energy must be utilised for maintenance (cell repair), metabolism, development of extra-embryonic tissues, physical activity and excretion. The effect of temperature on the GEG of teleosts has been assessed by several authors (Johns and Howell 1980, Heming 1982, Rombough 1988a), however similar research has not been reported directly for elasmobranch species.

Ricklefs and Cullen (1973) suggested that increased incubation periods in poikilotherms, as a direct result of lowered temperatures, increased the maintenance component and consequently reduced the efficiency of energy utilised for growth (Chapter 4, section 4.1.3, p. 99). If this were true, there would be a negative affect on the hatchling mass. Ackerman (1981a) and Vleck and Hoyt (1991) countered this by arguing that reduced temperatures effectively decreased the metabolic processes and hence reduced energy consumption, with no consequence on the hatchling energy content (and arguably the mass). The hypothesis that lower incubation temperatures extend the incubation period and significantly reduce the efficiency with which yolk is transformed into animal tissue in *H. portusjacksoni* was tested.

## **3.2 MATERIALS AND METHODS**

### **3.2.1 Effect of temperature on development**

Eggs were placed into aquaria at 10, 15, 18, 20 or 22°C ± 0.5°C. Over the four year study, a total of eight eggs were held at 10°C, 30 eggs at 15°C, 98 eggs at 18°C, 164 at 20°C and 50 at 22°C. Eggs were examined for evidence of development at selected intervals, as part of other experimental procedures within this study (e.g. staging, growth and energetics).

### **3.2.2 Growth and allometry**

In this study, the rate of growth at 18, 20 and 22°C was measured in three ways : (1) as the increase in the whole and yolk-free embryo wet mass over time, (2) the increase in whole and yolk-free embryo dry mass over time and (3) the increase in total length over time.

A total of 81 embryos (stages 4-15) were examined to measure growth; 60 of these were individuals used for the staging procedure (Chapter 2, section 2.2.5, p. 17) plus an additional 21 embryos. The effect of temperature on growth, incubation period and hatchling size was determined. At selected times during development, the egg capsules were opened with a razor blade and the embryo exposed. The egg contents were carefully placed into a tared glass petri dish. The egg capsule was weighed and the total embryonic length, wet and dry masses of both the embryo (whole and yolk-free) and external and internal yolk masses were determined. The embryo was killed by dissolving a lethal dose of the anaesthetic tricaine methanesulphonate (Sandoz MS-222) to one litre of seawater (ca. 1g/g of embryo) and adding it to the fluid surrounding the embryo. The embryo was then separated from the external yolk mass by cutting through the yolk stalk near the embryo. The cut ends were held closed so that no yolk was lost. After the whole embryo was weighed, the embryo was dissected to remove any internal yolk mass. Wet masses were recorded by weighing the embryo and yolk separately (to the nearest mg) on a Sartorius 1265 MP balance. Total embryonic length (to the nearest mm) was recorded with callipers. Dry masses were determined by drying either whole embryos or homogenous samples of yolk in an oven overnight at 70°C until a stable weight was recorded. These data were represented as mass (wet) versus length plots (for allometric analysis) as well as changes in embryo (yolk-free, wet and dry) and external and internal yolk (wet and dry) mass with time.

For embryos that were kept alive and used for other experiments (for example, respiration and ventilation), and mass was required, the following method was used. The masses of the external yolk and the embryo were determined by establishing a tare on the balance with a 10 cm deep dish of seawater. The yolk was weighed by placing it in contact with the bottom of the dish, while holding the embryo out of the water by the 2-3 cm long yolk stalk (Figure 3.1). Care was taken not to put any tension on the yolk stalk during this procedure by allowing considerable slack in the stalk. The embryo was then placed into the water and a total mass recorded (yolk + embryo + yolk stalk) in grams (to the nearest 0.01 g). The yolk stalk was considered to be part of the yolk mass. The embryo mass was recorded as the difference between total mass and the yolk mass. To verify the masses recorded, the balance was tared on the sea water plus embryo and yolk. The embryo was then carefully held out of the water and the embryonic mass recorded as a negative reading. This procedure was repeated three times and mean values obtained for all components. Embryos between stage 10 and 11 had delicate yolk stalks, and the method described above for weighing the embryo directly could not be used. Therefore only a total length (in mm) was recorded and the wet mass was derived from the mathematical relationship between the two.

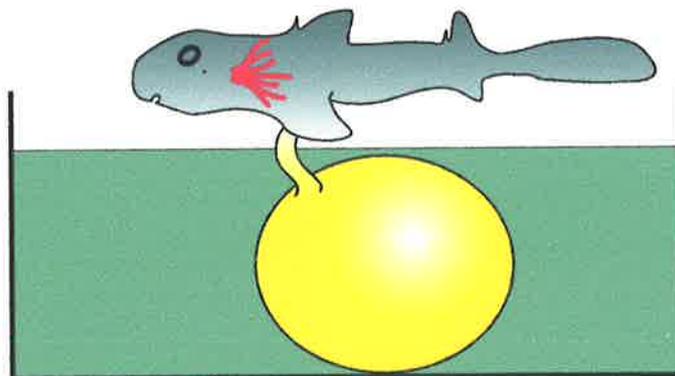


Figure 3.1 The method of measuring embryonic wet mass and yolk mass of live embryos (stage 12 and older). The embryo is held out of water while the yolk is supported on the balance.

### 3.2.3 Growth equations

The wet and dry masses of both whole and yolk-free embryos were plotted against incubation time and a least square fit for a logistic curve performed (Ricklefs 1967):

$$M_t = \frac{M_a}{1 + e^{-r(I - I_f)}} \quad (\text{eq. 3.1})$$

where  $M_t$  = the mass of the embryo at time  $t$  (g)

$M_a$  = the mass at the asymptote (g)

$e$  = base of natural logarithms

$r$  = growth constant

$I$  = incubation period (days)

and  $I_f$  = the incubation time at inflection point.

### 3.2.4 Growth rate

The rate of embryonic growth was determined by the following equation:

$$GR = \frac{Me(t_1) - Me(t_0)}{\text{time}(t)} \quad (\text{eq. 3.2})$$

where  $GR$  = the rate of growth (g/d)

$Me(t_1)$  = wet embryonic mass after a time interval  $t$  (g)

$Me(t_0)$  = wet embryonic mass at the start of interval  $t$  (g)

and  $\text{time}$  = the interval between mass increments (days).

The values of  $Me(t_1)$  and  $Me(t_0)$  at specific incubation intervals were determined from the derived logistic growth equations.

### 3.2.5 Rate of external yolk depletion

The wet masses of external yolk were plotted against incubation time. A least squares fit for a logistic curve was fitted to the data. The rate of yolk depletion was calculated with the following equation:

$$YDR = \frac{My(t_0) - My(t_1)}{\text{time}(t)} \quad (\text{eq. 3.3})$$

where  $YDR$  = the yolk depletion rate

$M(y)(t_0)$  = wet yolk mass at the start of interval  $t$  (g)

$M(y)(t_1)$  = wet yolk mass after a time interval  $t$  (g)

and  $\text{time}$  = the interval between yolk mass measurements (days).

The yolk depletion logistic curve was used to estimate yolk masses at selected intervals.

### **3.2.6 Gross efficiency of growth**

The percentage of dry tissue mass transferred from the yolk to the hatchling (GEG) was calculated as the ratio of the average dry mass of hatchling to the average predicted dry mass of yolk, multiplied by 100 (Marr 1966). It was impossible to measure the initial dry mass of external yolk of eggs from which individual embryos hatched directly, therefore it was necessary to predict the dry mass of yolk of successfully incubated eggs. Using known initial egg masses, the fresh wet yolk mass was estimated using equation 3.4 (chapter 3, p. 69). An estimate for yolk dry mass was determined from Table 4.1 (chapter 4, p.103), using the relationship between water content and fresh yolk mass.

### 3.3 RESULTS

#### 3.3.1 Allometry

##### 3.3.1.1 Relationship between initial egg mass and egg components

The components of fresh eggs were positively dependent on the initial egg mass (Figure 3.2). Linear regression analyses were performed on the data (equations 3.4-3.7). There were significant correlations between total initial egg mass and all components examined at the 1% level.

$$Y = 13.78 + 0.138 E_i \quad n=17 \quad r^2=0.56 \quad F=19.235 \quad (\text{eq. 3.4})$$

$$A = -2.026 + 0.419 E_i \quad n=17 \quad r^2=0.71 \quad F = 37.02 \quad (\text{eq. 3.5})$$

$$C = -6.042 + 0.414 E_i \quad n=28 \quad r^2=0.63 \quad F = 43.45 \quad (\text{eq. 3.6})$$

$$H = 24.263 + 0.208 E_i \quad n=31 \quad r^2=0.34 \quad F = 14.76 \quad (\text{eq. 3.7})$$

where  $Y$  = initial wet mass of yolk (g)

$A$  = initial mass of albumen (g)

$C$  = initial mass of egg capsule (g)

$H$  = wet hatchling mass (g)

and  $E_i$  = initial egg mass (g).

##### 3.3.1.2 Effect of initial egg mass on incubation period

At all temperatures, the length of incubation was not affected by the initial egg mass at the 1% level of significance (Figure 3.3). Linear regressions performed on these data produced the following equations:

$$18^\circ\text{C} \quad I = 36.638 + 0.319 E_i \quad n=8 \quad r^2=0.033 \quad F=0.201 \quad (\text{eq. 3.8})$$

$$20^\circ\text{C} \quad I = -9.136 + 0.538 E_i \quad n=12 \quad r^2=0.087 \quad F=0.948 \quad (\text{eq. 3.9})$$

$$22^\circ\text{C} \quad I = 221.98 - 0.210 E_i \quad n=12 \quad r^2=0.006 \quad F=0.056 \quad (\text{eq. 3.10})$$

where  $I$  = the incubation period (days)

and  $E_i$  = the initial egg mass (g).

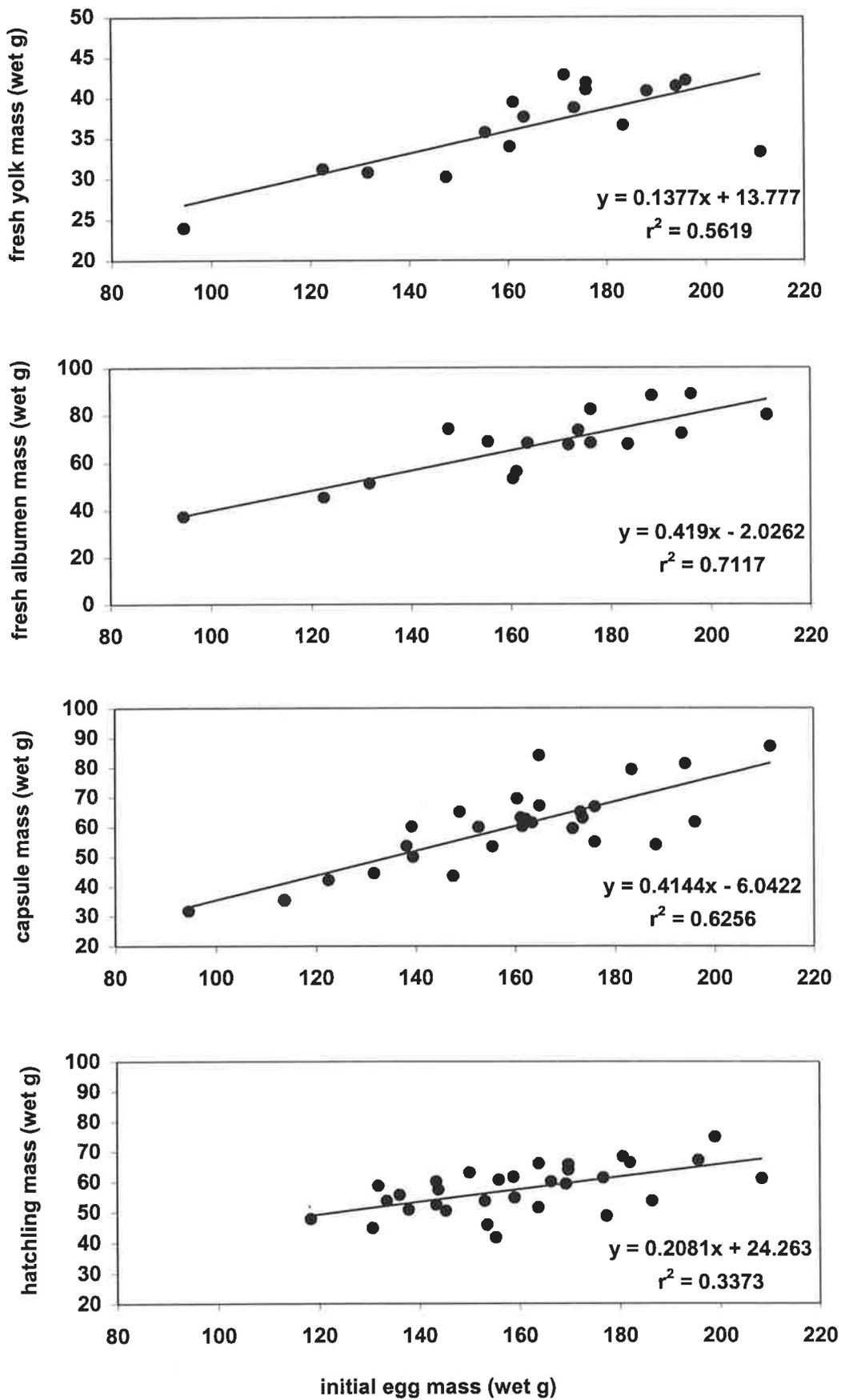


Figure 3.2 The relationship between initial egg mass and egg components.

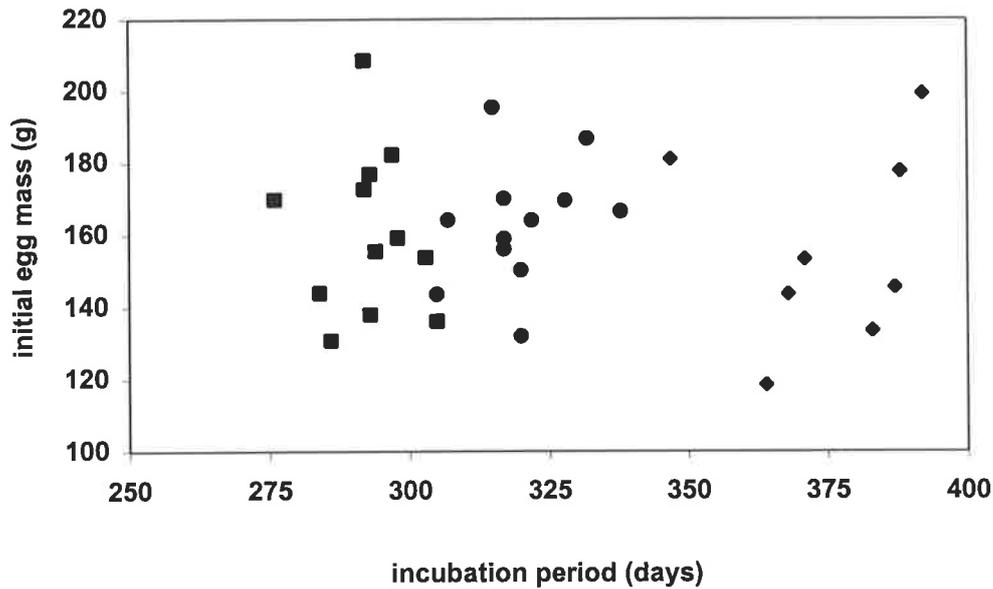


Figure 3.3 The effect of initial egg mass on incubation period at 18 (solid diamond), 20 (solid circle) and 22°C (solid square).

### 3.3.1.3 The relationship between embryo length and wet mass

The relationship between the total length and wet mass for yolk-free embryos is shown in Figure 3.4 (18, 20 and 22°C combined). Data from each temperature were taken and transformed into logarithmic functions described by the following equations:

$$18^{\circ}\text{C} \quad \log L = 1.148 + 0.307 \log M(\text{yf}) \quad r^2=0.996 \quad n=18 \quad (\text{eq. 3.11})$$

$$20^{\circ}\text{C} \quad \log L = 0.846 + 0.308 \log M(\text{yf}) \quad r^2=0.993 \quad n=36 \quad (\text{eq. 3.12})$$

$$22^{\circ}\text{C} \quad \log L = 0.863 + 0.303 \log M(\text{yf}) \quad r^2=0.992 \quad n=26 \quad (\text{eq. 3.13})$$

Combined temperatures produced the logarithmic equation:

$$\log L = 0.862 + 0.299 \log M(\text{yf}) \quad r^2=0.992 \quad n=80 \quad (\text{eq. 3.14})$$

where L = total length (mm)

and M(yf)= yolk-free wet mass of embryo (mg).

At each temperature, the value 0.33 fell well within the 95% confidence interval of the exponent, suggesting isometric growth.

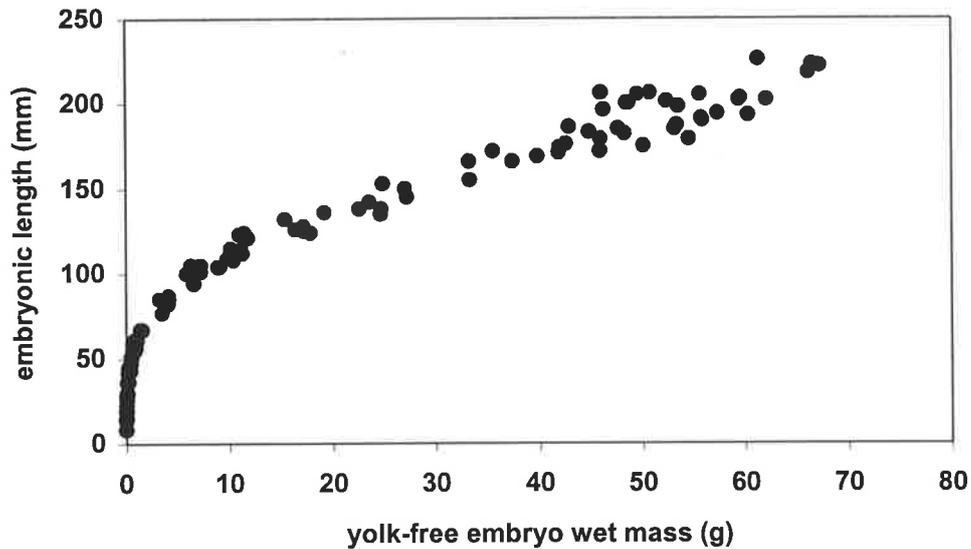


Figure 3.4 The relationship between total embryonic length and yolk-free embryo wet mass (all temperatures combined).

### 3.3.2 Effect of temperature on development

There was no sign of embryonic development or yolk vascularisation in eggs held at 10°C after 90 days of incubation. At 15°C, development was greatly repressed, with only two embryos (6.67%) reaching stage 11 before dying 270 days into the incubation, 135 days behind development at 20°C. Successful development occurred at incubation temperatures 18°C and above.

### 3.3.3 Effect of temperature on incubation period and hatchling size

Because the date of egg laying was unknown, the predictive nature of the opening date was used as a basis for calculation of the entire incubation period. To calculate the total incubation period, 120 days were added to the number of days between egg capsule opening and hatching (120 days representing the period between laying and egg capsule opening - see chapter 2, 2.3.5, p. 23). Table 3.1 describes the effect of experimental temperature on incubation time, hatchling total length, wet and dry whole mass and wet mass of the internal yolk of hatchlings.

Incubation time from capsule opening was clearly dependent upon the experimental temperature (ANOVA  $F = 304.918$ ,  $P < 0.001$ ). The data for initial egg mass only represented eggs from which hatchlings emerged. Initial egg mass was not significantly different at the three temperatures ( $F = 0.217$ ). This allowed the hatchling mass and

length to be directly compared between temperatures. There was no significant difference between hatchling wet mass, dry mass or total length at the three temperatures ( $F = 0.923, 2.68$  and  $0.674$  respectively) nor any significant difference in the amount of internal yolk left at hatching at the three temperatures ( $F = 0.447, P > 0.05$ ).

temperature	18°C	20°C	22°C
incubation period (days)	379.8 ± 3.64 (14)	321.4 ± 2.17 (17)	291.3 ± 1.82 (18)
total initial egg mass (g)	156.13 ± 9.58 (8)	162.95 ± 5.01 (12)	159.37 ± 7.04 (11)
hatchling mass (wet g)	56.85 ± 2.42 (14)	58.79 ± 1.65 (17)	54.91 ± 1.85 (17)
hatchling mass (dry g)	16.08 ± 0.88 (6)	17.34 ± 0.44 (12)	17.65 ± 0.81 (11)
hatchling total length (mm)	203 ± 2.45 (14)	207 ± 1.98 (17)	204 ± 2.95 (17)
hatchling internal yolk (wet g)	0.68 ± 0.34 (6)	0.42 ± 0.12 (6)	0.76 ± 0.29 (6)

Table 3.1 Effect of temperature on incubation and hatchling size. Data are mean ± standard error (n).

### 3.3.4 Changes in wet mass of yolk-free embryos and yolks (external and internal) during incubation

Figure 3.5 demonstrates changes in wet mass that occurred of the yolk-free embryo and the external and internal yolks as incubation proceeded at 18, 20 and 22°C. The external yolk remained relatively constant for the first 120 days at all temperatures, during which time, there was minimal production of animal tissue. The external yolk began to decrease in mass as a result of the significant production of animal tissue at around 150 days. After a further 60 days, the external yolk was greatly reduced by the embryo's developmental requirements and with the internalisation of yolk in an internal sac (stage 12). The external yolk was completely gone (stage 14) by around day 270, 250 and 230 at 18, 20 and 22°C respectively. Around this time, the internal sac reached

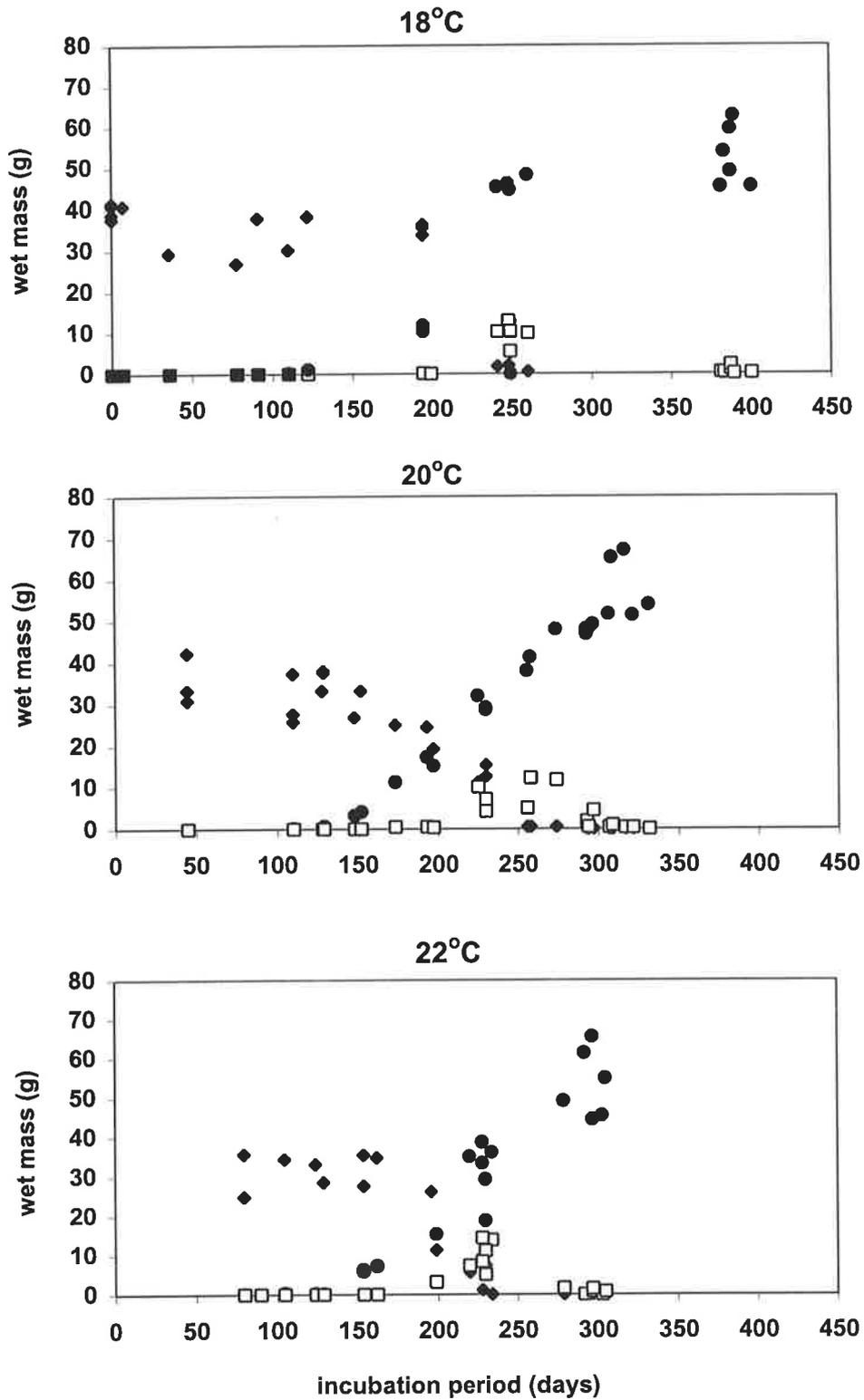


Figure 3.5 Changes in mass of yolk-free embryo (solid circle), external yolk (solid diamond) and internal yolk (open squares) during incubation at 18, 20 and 22°C.

maximal mass of approximately 12-15 g (wet). From this time until hatching, the embryo progressively increased in size as it used the internal yolk. Hatching occurred when the internal reserves were virtually gone (stage 15). The time that it took the embryo to use up internal yolk reserves and hatch differed between experimental temperatures. The higher the temperature, the quicker the reserves were depleted and the shorter the incubation period.

### 3.3.5 Growth

#### 3.3.5.1 Mass increments

The pattern of growth in wet whole mass (Figure 3.6) and yolk-free embryo mass (Figure 3.7) over time was weakly sigmoidal at 20 and 22°C, and more strongly sigmoidal at 18°C. Logistic equations were constructed using non-linear statistics, for wet and dry masses of both whole and yolk-free embryos.

#### Wet mass

$$18^{\circ}\text{C} \quad M_e = \frac{52.024}{1 + e^{-0.035(I-231.643)}} \quad r^2 = 0.99 \quad n = 24 \quad (\text{eq. 3.15})$$

$$18^{\circ}\text{C} \quad M_{yfe} = \frac{53.049}{1 + e^{-0.054(I-218.067)}} \quad r^2 = 0.99 \quad n = 24 \quad (\text{eq. 3.16})$$

$$20^{\circ}\text{C} \quad M_e = \frac{56.281}{1 + e^{-0.042(I-213.210)}} \quad r^2 = 0.99 \quad n = 33 \quad (\text{eq. 3.17})$$

$$20^{\circ}\text{C} \quad M_{yfe} = \frac{58.418}{1 + e^{-0.031(I-228.917)}} \quad r^2 = 0.99 \quad n = 33 \quad (\text{eq. 3.18})$$

$$22^{\circ}\text{C} \quad M_e = \frac{55.079}{1 + e^{-0.048(I-205.236)}} \quad r^2 = 0.97 \quad n = 24 \quad (\text{eq. 3.19})$$

$$22^{\circ}\text{C} \quad M_{yfe} = \frac{55.705}{1 + e^{-0.035(I-222.296)}} \quad r^2 = 0.97 \quad n = 24 \quad (\text{eq. 3.20})$$

#### Dry mass

$$18^{\circ}\text{C} \quad M_e = \frac{16.133}{1 + e^{-0.034(I-238.052)}} \quad r^2 = 0.98 \quad n = 24 \quad (\text{eq. 3.21})$$

$$18^{\circ}\text{C} \quad M_{yfe} = \frac{20.971}{1 + e^{-0.018(I-330.924)}} \quad r^2 = 0.97 \quad n = 24 \quad (\text{eq. 3.22})$$

$$20^{\circ}\text{C} \quad M_e = \frac{17.270}{1 + e^{-0.036(I-230.247)}} \quad r^2 = 0.99 \quad n = 33 \quad (\text{eq. 3.23})$$

$$20^{\circ}\text{C} \quad M_{yfe} = \frac{20.771}{1 + e^{-0.030(I-273.950)}} \quad r^2 = 0.96 \quad n = 33 \quad (\text{eq. 3.24})$$

$$22^{\circ}\text{C} \quad M_e = \frac{17.925}{1 + e^{-0.036(I-225.924)}} \quad r^2 = 0.97 \quad n = 24 \quad (\text{eq. 3.25})$$

$$22^{\circ}\text{C} \quad M_{yfe} = \frac{20.845}{1 + e^{-0.040(I-265.552)}} \quad r^2 = 0.95 \quad n = 24 \quad (\text{eq. 3.26})$$

where  $M_e$  = the whole dry embryo mass including internal yolk (g),  
 $M_{yfe}$  = the dry mass of the yolk-free embryo (g)  
and  $I$  = the period of incubation (days).

Examination of the effect of temperature on growth was made for data between 150 and 280 days of incubation where growth was approximately linear. Linear regressions were performed on time versus wet embryo mass (whole and yolk-free) and the slopes of these data were compared with an ANCOVA analysis. ANCOVA analyses showed that there was no significant difference between slopes for whole and yolk-free embryos at the 1% level at the three temperatures ( $F= 0.628$  and  $F= 0.338$  respectively). The linear regression analysis produced the following equations:

$$18^{\circ}\text{C} \quad M(e) = -99.863 + 0.574 I \quad r^2=0.926 \quad n=8 \quad (\text{eq. 3.27})$$

$$M(yfe) = -67.721 + 0.406 I \quad r^2=0.946 \quad n=8 \quad (\text{eq. 3.28})$$

$$20^{\circ}\text{C} \quad M(e) = -68.928 + 0.459 I \quad r^2=0.943 \quad n=10 \quad (\text{eq. 3.29})$$

$$M(yfe) = -51.278 + 0.355 I \quad r^2=0.978 \quad n=10 \quad (\text{eq. 3.30})$$

$$22^{\circ}\text{C} \quad M(e) = -71.564 + 0.493 I \quad r^2=0.805 \quad n=10 \quad (\text{eq. 3.31})$$

$$M(yfe) = -49.344 + 0.353 I \quad r^2=0.796 \quad n=10 \quad (\text{eq. 3.32})$$

where  $M(e)$  = the wet mass of the whole embryo (grams)  
 $M(yfe)$  = the wet mass of the yolk-free embryo (grams)  
and  $I$  = incubation (days).

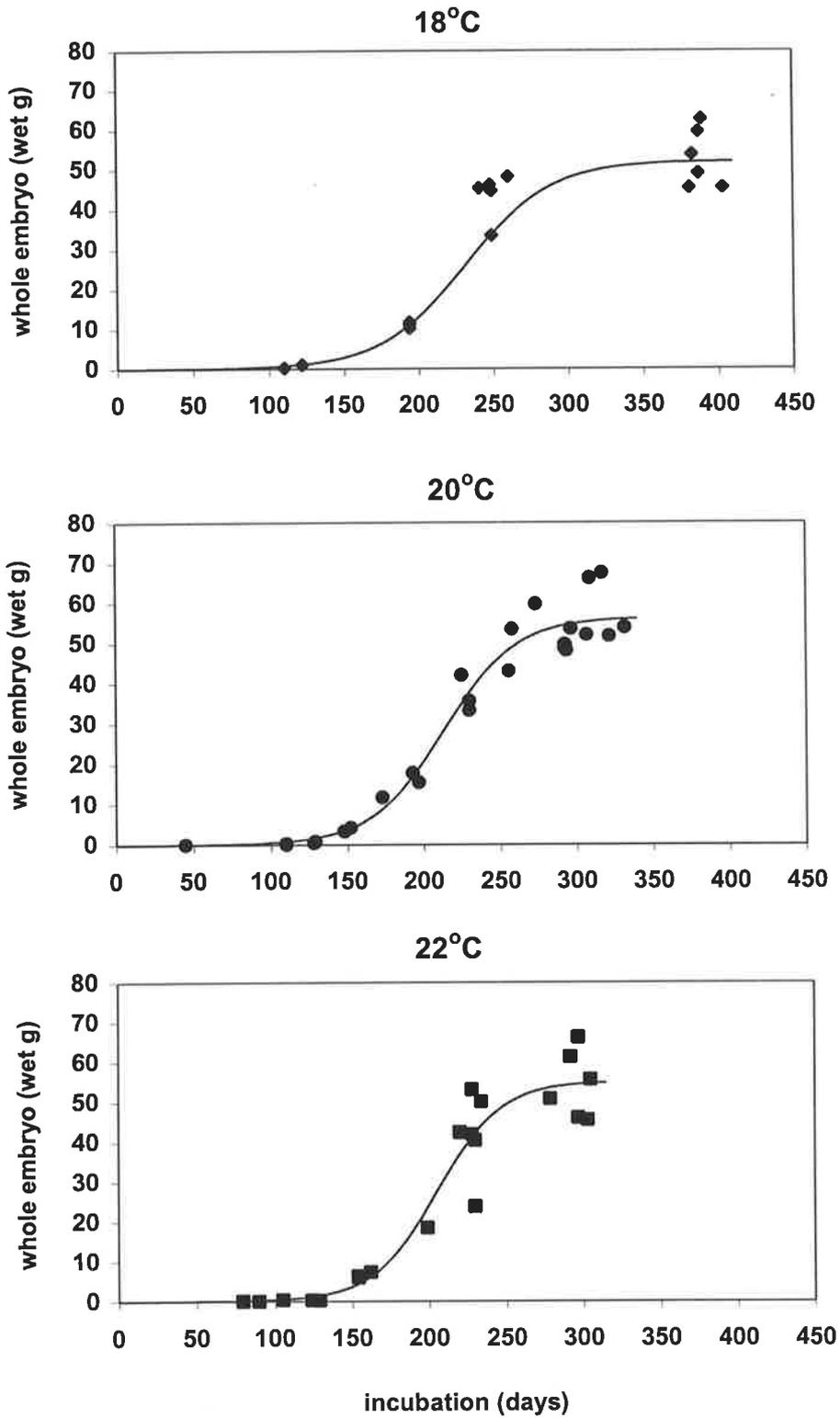


Figure 3.6 Pattern of growth (expressed as increasing wet whole mass) in embryos at 18, 20 and 22°C throughout incubation.

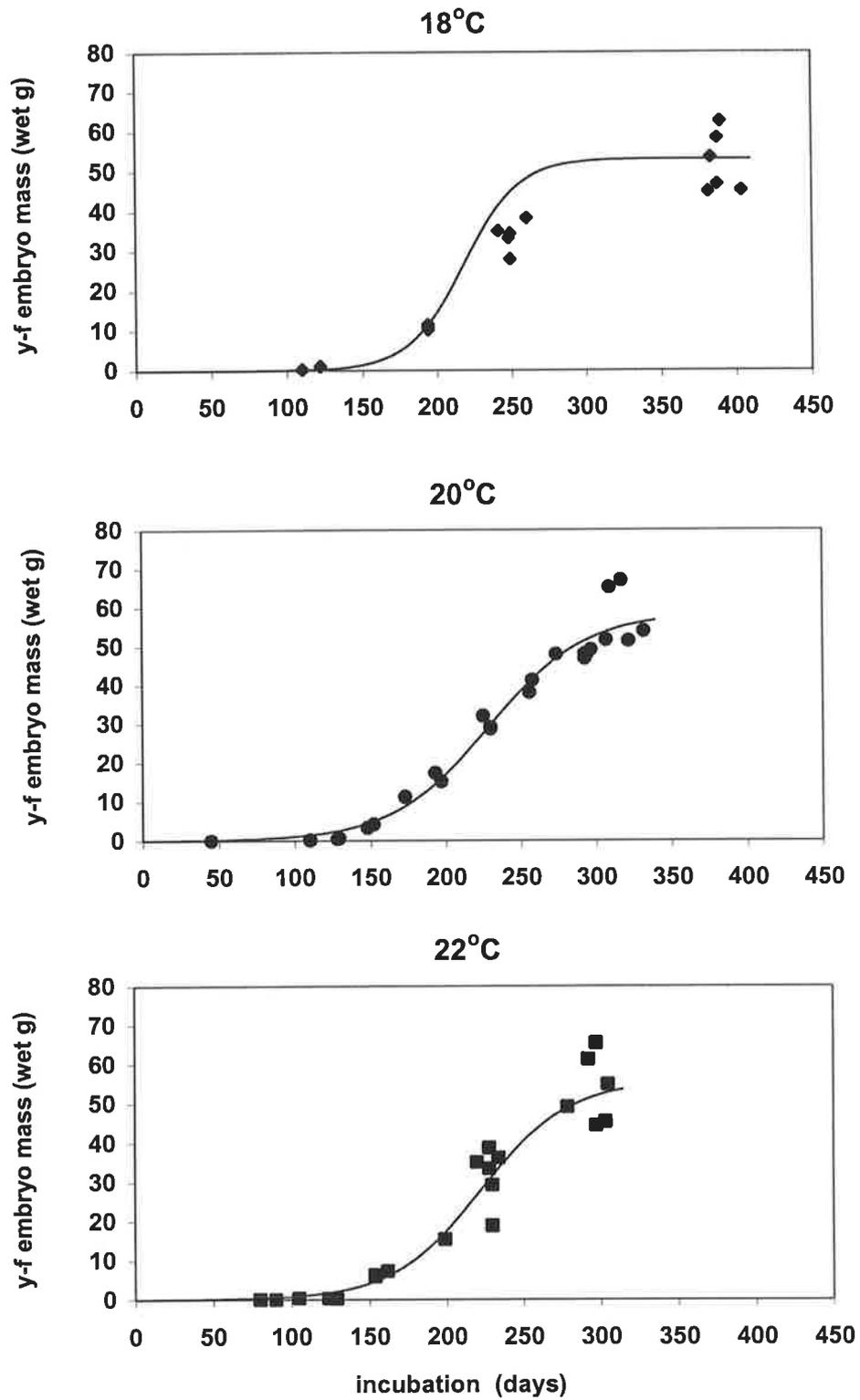


Figure 3.7 Pattern of growth (expressed as increasing yolk-free wet mass) in embryos at 18, 20 and 22°C throughout incubation.

### 3.3.5.2 Length increments

Embryonic length through time increases in a weak sigmoidal pattern (Figure 3.8). The lower the incubation temperature, the more pronounced the sigmoidal pattern. The polynomial equations below best described the data:

$$18^{\circ}\text{C} \quad L = -1\text{E-}05\text{I}^3 + 0.0048\text{I}^2 + 0.272\text{I} - 34.9.9 \quad r^2=0.984 \quad n=18 \quad (\text{eq. 3.33})$$

$$20^{\circ}\text{C} \quad L = -2\text{E-}05\text{I}^3 + 0.0113\text{I}^2 - 0.9008\text{I} + 30.454 \quad r^2=0.990 \quad n=36 \quad (\text{eq. 3.34})$$

$$22^{\circ}\text{C} \quad L = -2\text{E-}05\text{I}^3 + 0.0106\text{I}^2 - 0.6539\text{I} + 14.235 \quad r^2=0.966 \quad n=26 \quad (\text{eq. 3.35})$$

where L = total embryonic length (mm)

and I = incubation (days).

### 3.3.5.3 Growth rate

The rate of growth (wet g per day) was calculated for each temperature (Table 3.2). The data show that the rate of growth was slow during the first 120 days for all temperatures, the embryo amassing less than 2% of final hatchling mass at 40% of the incubation. An increase in the growth rate was observed around 120 days, coinciding with the opening of the egg capsule (Figure 3.9). Maximum growth rates of 0.45, 0.579 and 0.577 g/d were observed at 62%, 63% and 68% of incubation at 18, 20 and 22°C respectively. Thereafter, the growth rate decreased to 3% of the maximum rate at hatching.

GROWTH RATE			
time (d)	18°C	20°C	22°C
20	0.001	0.001	0.001
60	0.005	0.004	0.004
100	0.019	0.022	0.02
130	0.049	0.069	0.069
150	0.095	0.147	0.166
170	0.171	0.286	0.35
190	0.279	0.467	0.573
210	0.393	0.579	0.577
230	0.45	0.518	0.472
250	0.408	0.344	0.252
270	0.299	0.185	0.111
290	0.187	0.089	0.045
310	0.105	0.04	0.018 h
330	0.056	0.018 h	
350	0.029		
370	0.014 h		

Table 3.2 Growth rate (wet g per day) throughout incubation, as determined from the logistic growth equations. h = hatched.

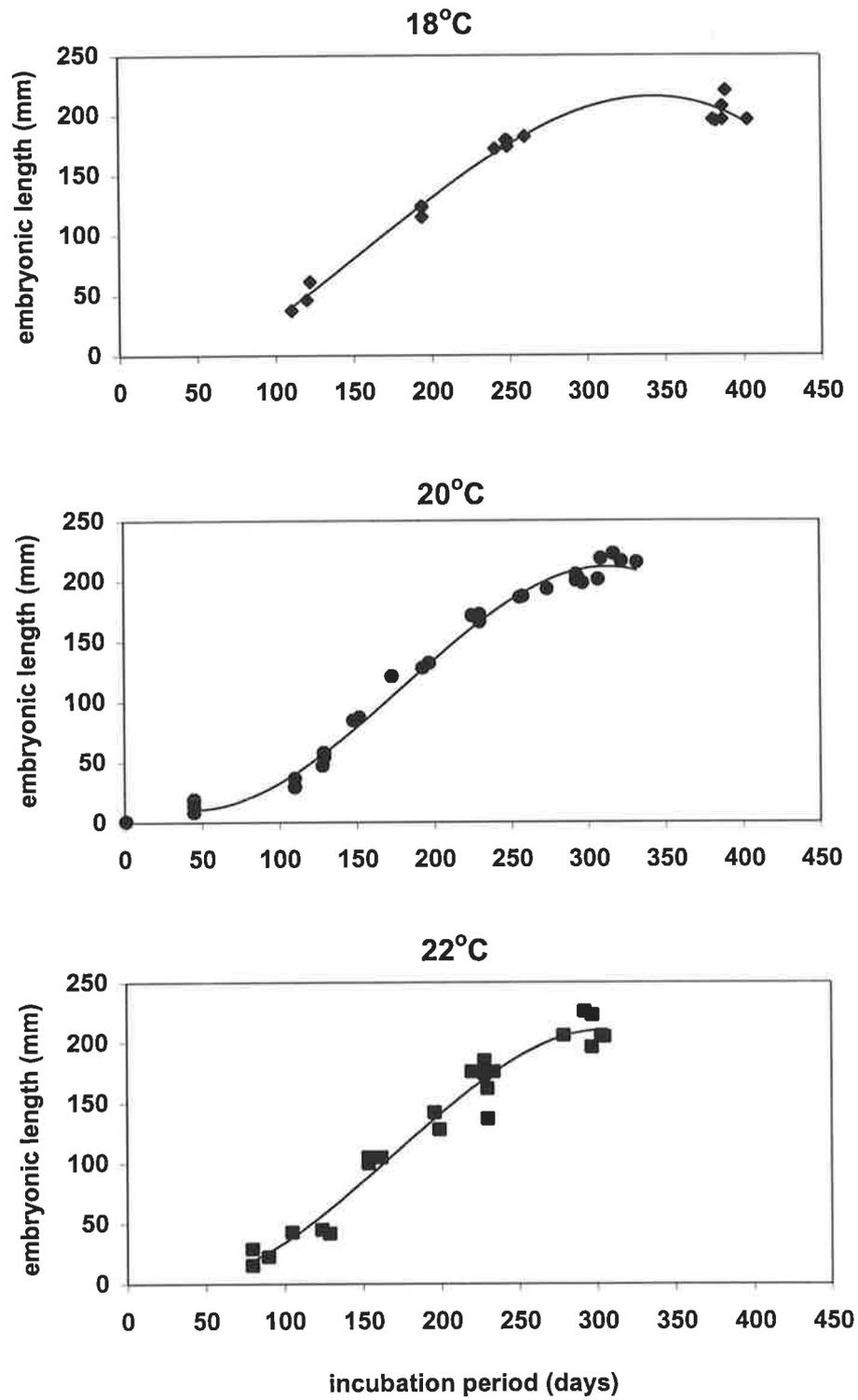


Figure 3.8 Pattern of growth (expressed as increasing length) in embryos at 18, 20 and 22°C throughout incubation.

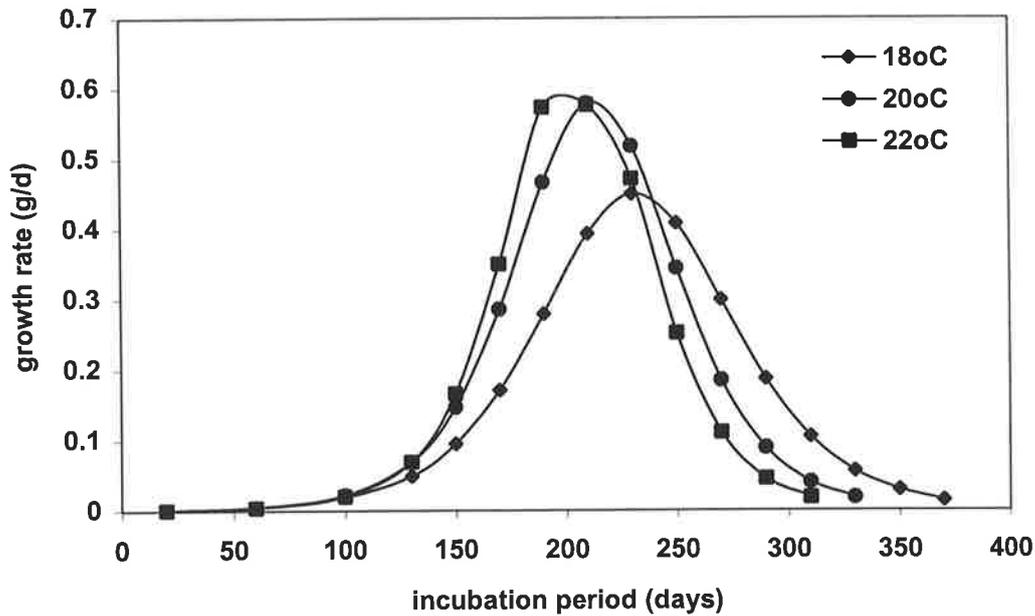


Figure 3.9 Growth rate (calculated from growth equations) at 18, 20 and 22°C.

#### 3.3.5.4 Effect of temperature on yolk absorption rates

The pattern of external yolk depletion was reverse sigmoidal (Figure 3.10) and described by the logistic equations below.

$$18^{\circ}\text{C} \quad Y = \frac{39.954}{1 + e^{0.03(I-179.339)}} \quad r^2 = 0.99 \quad n = 19 \quad (\text{eq. 3.36})$$

$$20^{\circ}\text{C} \quad Y = \frac{36.371}{1 + e^{0.036(I-202.572)}} \quad r^2 = 0.98 \quad n = 33 \quad (\text{eq. 3.37})$$

$$22^{\circ}\text{C} \quad Y = \frac{35.977}{1 + e^{0.054(I-197.431)}} \quad r^2 = 0.97 \quad n = 25 \quad (\text{eq. 3.38})$$

where Y = the wet mass of external yolk  
and I = number of days of incubation.

The rate of external yolk depletion was calculated using equations 3.36-3.38. The logistic growth (or depletion) constants show an increasing trend with increasing temperature (0.03, 0.036, and 0.054 at 18, 20 and 22°C respectively).

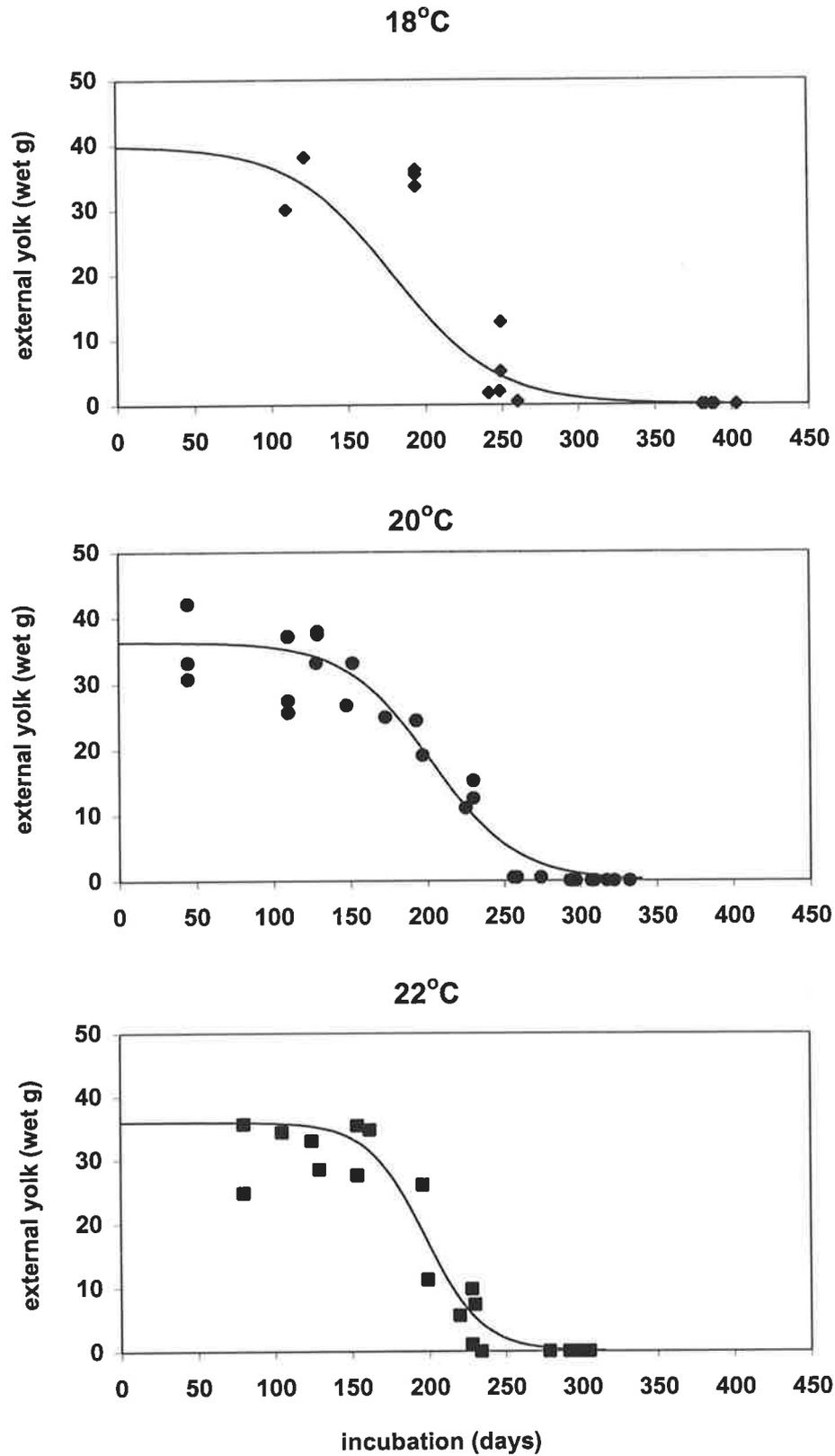


Figure 3.10 Pattern of external yolk depletion over time at 18, 20 and 22°C.

### 3.3.6 Gross efficiency of growth

The relationship between initial wet yolk mass and initial egg mass (eq. 3.4, p. 69) was used to calculate the individual initial wet yolk mass for each egg from which a hatchling emerged. The dry mass of the yolk was calculated from Table 4.1 (chapter 4, p.103) which related wet yolk mass and water content. The dry mass of individual hatchlings was known directly (by drying in an oven) or calculated from Table 4.2 (chapter 4, p. 104) relating water content and wet hatchling mass. The results are presented in Table 3.3 as mean  $\pm$  standard errors (n). There was no significant difference in GEG between temperatures (ANOVA F= 2.614).

<b>temperature</b>	<b>18°C</b>	<b>20°C</b>	<b>22°C</b>
<b>yolk mass (dry g)</b>	20.70 $\pm$ 0.924 (6)	21.12 $\pm$ 0.367 (12)	20.69 $\pm$ 0.571 (10)
<b>hatchling mass (dry g)</b>	16.08 $\pm$ 0.88 (6)	17.34 $\pm$ 0.44 (12)	17.65 $\pm$ 0.81 (11)
<b>GEG</b>	<b>77.7</b>	<b>82.1</b>	<b>85.3</b>

Table 3.3 The effect of experimental temperature on gross efficiency of growth, defined as the ratio of the increase in dry weight of the yolk-free embryo to the decrease in dry weight of the initial yolk, multiplied by 100.

### **3.4 DISCUSSION**

#### **3.4.1 Allometry**

There are a number of significant relationships between variables observed in the development of *H. portusjacksoni* embryos. The initial egg mass was positively correlated with all egg components and the size of hatchling (Figure 3.2, p. 70). The relationships between initial egg mass and egg contents are useful in estimating unknown variables from known ones. For instance, if the mass of the fresh egg is known then the initial yolk mass can be calculated. This then becomes useful in estimating the dry mass of yolk (Table 4.1, chapter 4, p. 103) and hence GEG if the dry mass of the hatchling is known. Such estimates should be treated with some care however, because the accuracy of prediction is reduced with every progressive assumption.

##### **3.4.1.1 Effect of initial egg mass on incubation period**

The present study reported no significant effect of egg mass on the incubation period of *H. portusjacksoni* in any of the three temperatures investigated despite the large range of egg masses used (118.1-208.1 g) (Figure 3.3, p.71). The absence of such a relationship is the result of the effect of larger eggs producing larger embryos that consume more oxygen, and utilise yolk at a greater rate than smaller embryos. The overall result is that the yolk is depleted over similar periods, hence incubation period is unaffected.

An intra-specific relationship between fresh egg mass and incubation period has rarely been investigated because the natural variation in egg mass is often small within a species. Over a range of egg masses from 150-400 mg of chum, *Orcorhynchus keta*, there was no significant correlation with incubation period (Hayashizaki et al. 1995). The size of salmon eggs, *S. salar*, also had no effect on the length of incubation (Kazakov 1981). Pepin (1991) reviewed the available literature on teleost incubation and found a general trend showing a significant increase in the incubation period with larger eggs, based on data grouped from closely related species. More specifically, egg diameter had no effect on developmental times during the egg phase, but significantly effected the period between hatching and total yolk absorption (Pepin et al. 1997).

##### **3.4.1.2 Effect of initial egg mass on hatchling size**

Hatchling mass is positively correlated with fresh egg mass in *H. portusjacksoni* (Figure 3.2, p. 70). This is also observed in teleosts (Kazakov 1981, Wallace and

Aasjord 1984, Escaffre and Bergot 1984, Knutsen and Tilseth 1985, Springate and Bromage 1985, Beacham and Murray 1990), reptiles (Yntema 1968, Ewert 1985, Deeming and Ferguson 1989, Whitehead et al. 1992) and birds (Whitehead et al. 1990, Wilson 1991). Beacham and Murray (1990) found that the egg mass was the major controlling factor in the resultant mass and length of fry in five species of Pacific salmon.

The mass attained by a hatchling fish is limited by the initial yolk mass. All eggs have a yolk store sufficient to ensure adequate development. Larger eggs, with greater yolk supplies, generally produce larger hatchlings which are better prepared to survive adverse conditions for longer than smaller offspring (Kazakov 1981). However, any size advantage is lost after the start of exogenous feeding in the teleost larvae *Salmo gairdneri* and *Salmo salar* (Thorpe et al. 1984, Springate and Bromage 1985). The advantage of larger size at hatch in larger bird eggs is also shown to be lost within one week in European starlings, *Sturnus vulgaris* (Smith et al. 1995).

#### **3.4.1.3 Length-mass relationship**

In many fish-based growth studies, length is the preferable variable because it is easier to measure, particularly at sea where weighing is difficult. Mass measurements are also difficult to measure in small embryos so length is a suitable growth determinant where a good relationship exists between body mass and length (Treasurer 1976, Keckeis and Schiemer 1990). The substitution of total length measurements to estimate body mass of small embryos reduces the stress due to handling, particularly out of water.

Growth in *H. portusjacksoni* embryos, as observed as the relative changes in length with wet mass, is isometric, that is, the embryo stays the same shape from stage 4 onwards (eq. 3.14, slope = 0.299, p. 71). This isometric relationship is commonly assumed for adult fish, but growth in embryonic teleosts has been shown to deviate from this (Lasker et al. 1970, Ehrlich et al. 1976, Laurence 1979). Slopes closer to 4 were found for herring, *Clupea harengus*, plaice, *Pleuronectes platessa*, and several species of flounder (Ehrlich et al. 1976, Laurence 1979), suggesting that the dry mass of larval fish may be more closely proportional to length to the fourth power rather than cubed.

### 3.4.2 Effect of temperature on development

This study demonstrated no development at low temperatures (10°C), and limited development at 15°C in *H. portusjacksoni* embryos. Under laboratory conditions of constant temperature, extended exposure to 15°C led to a failure to continue development and all embryos died before or just after egg capsule opening (stage 11). Presumably the cells could not continue to function at the reduced rate enforced by the conditions of low temperature and the embryos died. Complete development was successful at 18°C. Therefore, the lower thermal limits for embryonic development in this species lie between 15 and 18°C. With the exception of one particularly warm measurement, sea temperatures at the time of egg laying were 14-16°C (Appendix 1, Fig. 7, p. 187). By the time there was observable growth (stage 4) the sea temperatures rose above 16°C and near the lower limit of tolerance. Growth was not examined at temperatures above 22°C, not allowing an upper thermal limit for development to be determined for this species. However, natural variations in temperature over two years show that it is not likely to reach above 22°C (Appendix 1, Fig. 7, p. 187). Hence, the temperature range for normal development in *H. portusjacksoni* was 18-22°C.

Normal development of embryos generally occurs within a specific temperature range, beyond which development is retarded (Swift 1964, Wang et al. 1987, Lang et al. 1994, Gibson and Johnston 1995) or completely arrested (Rajagopal 1979, Pipe and Walker 1987). The range of temperature tolerances among poikilothermic species differ and is partly determined by the thermal incubation history of the species (Volpe 1957). Over the entire incubation period, it is likely that the developing embryo experiences a range of environmental temperatures, either on a daily or seasonal basis. If the embryo is to develop successfully, it must be able to cope with the temperature fluctuations. Limits of thermal tolerances are generally not examined in elasmobranch studies with experiments generally performed at a single naturally occurring temperature. However it was found that clear-nosed skates, *Raja eglanteria*, were unable to develop at temperatures above 24°C (Libby and Gilbert 1960). In contrast, upper and lower limits of thermal tolerances have been studied extensively in teleosts (Brett 1951, Swift 1964, Rajagopal 1979, Pipe and Walker 1987, Wang et al. 1987). In some studies, the early teleost developmental stages were more sensitive to variations in temperature than yolk-sac larvae (Alderdice and Forrester 1971, Wang et al. 1987, Pepin 1991). Pre-hatch stages of Atlantic salmon, *S. salar*, developed successfully within a smaller temperature

range than hatched larvae (Ojanguren et al. 1999). The optimum temperature for growth may also change in different stages of development, with yolk-sac embryos having either no optimum temperature for growth or a lower one compared to embryos that have absorbed their yolk-sac (Zwiefel and Lasker 1976, Rajagopal 1979). Thermal tolerances for normal development of most amphibian embryos lie between 15-20°C, with some (*Bufo valliceps*) tolerating wider variations in temperature of 18-35°C (Volpe 1957). In some species, the younger developmental stages have greater heat resistance and broader thermal tolerances than older larvae (Duellman and Trueb 1986).

### 3.4.3 Effect of temperature on incubation period

The effect of experimental temperature on the incubation period in *H. portusjacksoni* was highly significant (Table 3.1, p. 73). The incubation period was reduced as the incubation temperature increased as a consequence of temperature effects on metabolic rate, yolk utilisation and embryonic growth. The higher the temperature, the greater the rate of yolk utilisation, and the earlier the embryo hatches. The inverse relationship between temperature and incubation periods in teleost eggs has been widely reported (Marr 1966, Ryland and Nichols 1967, Rajagopal 1979, Herzig and Winkler 1986, Pipe and Walker 1987, Miranda et al. 1990, Canino 1994, Gibson and Johnston 1995). This relationship exists in all ectothermic groups (Yntema 1968, Ewert 1985, Hubert 1985, Deeming and Ferguson 1989, Seymour et al. 1991), as a direct result of temperature on metabolic processes in ectothermic animals. Within the normal temperature range for development, the incubation period showed a curvilinear relationship with temperature in three teleost species studied by Herzig and Winkler (1986) and in sardine eggs (Miranda et al. 1990). Hatching occurred 2.7 times earlier at 16.2°C than at 10.9°C in bream, *Abramis brama*, while hatching was hastened by 5.5 and 2.9 fold in *Chalcalburnus chalcoides mento* and *Vimba vimba* at increased incubation temperatures (Herzig and Winkler 1986). Development at 20°C occurred approximately 3 times faster than at 11°C in sardine eggs, *Sardina pilchardus* (Miranda et al. 1990). A 10°C increase in incubation temperature resulted in hatching 3-4 times sooner in scad eggs, *Trachurus trachurus* (Pipe and Walker 1987), while development took half the time at 16°C than at 12°C for turbot embryos, *Scophthalmus maximus* (Gibson and Johnston 1995).

#### 3.4.4 Effect of temperature on hatchling size

Results from this study report no significant difference in hatchling mass (wet and dry) or in the mass of internal yolk at the three temperatures despite an overall increase of 90 days of incubation at the lower temperature (Table 3.1, p.73). Increased temperature quickens the incubation process, but the effect on the resultant hatchling size among teleosts is inconstant in the literature. Howell (1980) reported intermediate incubation temperatures of 8 and 10°C produced the largest yolk-sac larvae in the teleost, *Limanda ferruginea*, while extreme temperatures of 4 and 12°C resulted in smaller larvae. The smaller larvae did however have the advantage of larger yolk-sacs, and by the time the yolk was completely absorbed, the size difference was insignificant. The extreme temperatures of 4 and 12°C may have been outside the thermal range for the species and development was adversely affected. A positive linear relationship between temperature and body size (length and mass) was reported in the early stages of Atlantic salmon, *S. salar*, however it was reversed in yolk exhausted stages, with increasing temperature producing smaller larvae (Ojanguren et al. 1999). Johns and Howell (1980) showed no significant difference in the mass of flounder larvae, *Paralichthys dentatus*, reared at 16 and 21°C. Larval size and yolk-sac volume at hatching of walleye pollock, *Theragra chalcogramma*, were inversely related to incubation temperature (Canino 1994). The mean standard lengths of pollock larvae raised at 9°C and 3°C were 3.74 and 4.74 mm respectively, although the data suggested premature hatching at the higher temperature. Smaller hatchlings at higher incubating temperatures are also reported by Garside (1966), Wang et al. (1987) and Arul (1991). Shortened incubation periods in teleost fish appear to have conflicting effects on the developmental state of the larvae. Wang et al. (1987) reported that hatchlings emerged sooner at higher temperatures but were less developed. Rombough (1988a) found that steelhead hatchling size was unaffected by incubation temperature in the range of 6-12°C. However at 15°C the hatchlings were smaller and less advanced in their development. In contrast Collins and Nelson (1993) found that accelerated development of coral-reef fish, *Siganus randalli*, at higher temperatures produced larvae of advanced developmental state but with less yolk reserves. The trade-off for accelerated development was less reserves and a greater chance of starvation.

The effect of increasing incubation temperature on the hatchling mass in reptiles is also inconsistent. Incubation at higher temperatures increased the rate of development at the

expense of the hatchling mass in *Alligator mississippiensis* (Deeming and Ferguson 1989). However the smaller hatchling alligator does have a larger yolk reserve. In contrast, higher temperatures produce larger hatchlings in the smooth softshell turtle, *Apalone mutica*, (Janzen 1993) although internal reserves at hatch were not reported and may have been a significant proportion of the hatchling mass. In amphibians and reptiles, the volume of internal yolk supplies at hatch is determined by the incubating conditions (Deeming and Ferguson 1989, Seymour et al. 1991). Under conditions of extended incubation periods (as would occur if the temperature was lowered), the resultant hatchling may be larger as a result of more time to utilise the available yolk. Consequently, internal stores are reduced. In contrast, at higher incubation temperatures, growth is accelerated and incubation significantly shortened. Hatchlings may emerge smaller but have larger internal yolk stores. Although the mass of yolked and yolk-free hatchlings of the Brisbane river turtle, *Emydura signata*, did not vary significantly over incubation temperatures of 24-32°C (Booth 1998).

Increasing temperature clearly decreases the incubation period in ectothermic animals, but from the above discussion, general trends relating incubation temperature and hatchling size can not be made for fish or reptiles. Avian development occurs over a fairly tight temperature range and it is difficult to include birds in this discussion. The hypothesis is further confounded by the differences in yolk energy available for the different developmental modes (altricial through to precocial), making generalisations about the yolk store remaining at hatch and the mass of hatchlings difficult. In many of the studies reported above, it appears that the hatchling is smaller, but the yolk supplies are greater as incubation is hastened. In this study there was no change in hatchling mass or internal yolk stores, suggesting that the available yolk is simply used quicker at higher temperatures.

### **3.4.5 Growth Patterns**

Growth in *H. portusjacksoni*, as measured by mass (Figures 3.6, 3.7, p.77-78) over time, was weakly sigmoidal at 20 and 22°C, and more strongly sigmoidal at 18°C. Sigmoidal growth in *H. portusjacksoni* shows that growth is minimal in the first 120 days, while the egg capsule is still closed (the embryo is less than 2% of final hatchling mass). When the egg capsule opens, the embryo's growth may be stimulated by the increased aerobic capacity (chapter 5, section 5.4.2, p. 161), resulting in an exponential

accumulation of mass, however the growth rate slows down in the last 40% of incubation. The plateau phase at the end of incubation in species exhibiting sigmoidal growth may be the result of a failure to maintain high growth rates because the metabolic demands are limited by the inability to transport sufficient oxygen from the environment to the embryo. This may be caused by several factors such as reduced oxygen availability, low diffusion or convection through the egg environment (Metcalf et al. 1984, Paganelli and Rahn 1984, Whitehead 1987). It has also been hypothesised that the plateau may be the result of increased synthesis efficiency which leads to energy savings for synthesis costs, and a reduction of growth rate plays only a minor role (Dietz et al. 1998).

Environmental hypoxia has been correlated with reduced growth in teleost and avian embryos. An increase in the metabolic rate upon breaking free of the egg capsule is seen in many teleost (Holliday et al. 1964, Eldridge et al. 1977, Davenport and Lönning 1980, Davenport 1983, Rombough 1988b, Walsh and Lund 1989) and amphibian (Seymour and Bradford 1995) embryos. Similarly, oxygen demands in avian species may be greater than the shell conductance will cater for (Pettit and Whittow 1985). In fact, the late incubation embryo has been shown to be hypoxic prior to pipping. On pipping, there is a marked increase in the mass-specific metabolic rate of embryos, supporting limiting shell diffusion (Pettit and Whittow 1985, Vleck and Bucher 1998). The depressed metabolic rate prior to these events (pipping or hatching), due to the hypoxic conditions, directly inhibits the growth of the embryo (Gruber and Weiser 1983).

In the case of reptilian embryos, it has been shown that oxygen consumption rates are not altered significantly at pipping or hatching (Thompson 1983, Whitehead 1987). It is more likely to be the accumulation of excretory products such as urea that are debilitating on the growth rate (Ferguson 1985, Whitehead 1987).

The plateau phase demonstrating reduced growth exhibited by *H. portusjacksoni* is unlikely to be the result of environmental hypoxia or a build up of excretory product because there are no physical barriers to these substances because of the open flow nature of the egg capsule. At the time when the growth begins to slow down (230 days of incubation), the *H. portusjacksoni* embryo has reached a developmental stage at

which it is nearly complete (stage 14). There is no need to increase the mass of the embryo, just to complete development.

Elasmobranch growth patterns have generally been assessed by examining the increase in embryonic length with time. By this measurement, a weak sigmoidal growth pattern was recorded for *H. portusjacksoni* (Figure 3.8, p.80). Similarly, the ray, *R. brachyura*, (Clark 1926) and the Pacific angel shark, *S. californica* (Natanson and Cailliet 1986) demonstrated weak sigmoidal patterns of growth. The sand tiger shark, *O. taurus*, demonstrates a more marked sigmoidal pattern (Gilmore et al. 1983). Pelster and Bemis (1991) described growth of the embryonic little skate, *Raja erinacea*, as exponential. Length versus age until hatching was linear in embryonic white spotted bamboo shark, *Chiloscyllium plagiosum*, (Tullis and Peterson, 1997), however growth, as measured by mass, was sigmoidal. Rombough (1988a) also reports sigmoidal growth in embryos and larvae of steelhead embryos, *S. gairdneri*, from fertilisation to 90% yolk absorption.

#### **3.4.6 External yolk absorption rates**

The results from this study demonstrated that external yolk was depleted in an inverse pattern to that observed for growth (Figure 3.10, p. 82). While the animal tissue mass remained low (up to day 120), the yolk mass remained fairly constant. Once embryonic mass began to accelerate, the external yolk was absorbed quickly, particularly through the rapid growth phase (day 150- 280), and once internalisation of the yolk began (Figure 3.5, p. 74). Complete external yolk exhaustion occurred around day 230-270 of incubation at a temperature range of 18-22°C. Internal yolk stores were maximal near the time external supplies were diminished. The internal yolk provided the energy for the remaining portion of incubation; for maintenance and any developmental processes that were still incomplete.

Temperature affected the external yolk absorption rate, with higher temperatures increasing the rate of yolk exhaustion, a result of the greater metabolic rates. Growth constants derived from the logistic curve for yolk absorption show an increasing trend with increasing temperature (0.3, 0.36 and 0.54 at 18, 20 and 22°C respectively - eq.s 3.33-3.35, p. 79). Internal yolk stores were also affected by temperature, reflected in the loss of internal yolk stores at 370, 300, and 370 days at 18, 20 and 22°C respectively (Figure 3.5, p. 74). Complete exhaustion of yolk supplies has great significance on the

total incubation period, because the energy reserves were removed at temperature dependent rates, and hatching was dependent upon the loss of these reserves.

The embryonic growth rate and the subsequent mass attained by a hatchling fish during incubation are determined by the initial quantity of yolk, the rate of yolk absorption and the efficiency with which the yolk is converted into tissue mass. The rate and efficiency of yolk absorption is influenced by many environmental factors. The efficiency with which an embryo transforms yolk into tissue is important because the greater amount of yolk successfully converted results in larger embryos.

The nutrients within the yolk are absorbed through the phagocytosis within the yolk syncytium, the inner most layer of the yolk membrane. The rate of absorption of yolk constituents and therefore growth depends upon the activity of the syncytium and its surface area, at least until the yolk stalk becomes functional. Temperature has the most profound effect on the rate of yolk utilisation (Garside 1966, May 1974, Santarre 1976, Hamor and Garside 1977, Howell 1980, Johns and Howell 1980, Heming 1982).

Within the normal temperature range, the absorption of yolk increases at higher temperatures in *Channo striatus* (Arul 1991), *Theragra chalcogramma* (Canino 1994), *Sardina pilchardus* (Miranda et al. 1990) and *S. salar* (Ojanguren et al. 1999), resulting in rapid growth and reduced time to hatch. Efficiency of absorption towards growth becomes reduced towards the upper and lower limits of the thermal range (May 1974, Wang et al. 1987). Increased temperature increases the activity of the yolk syncytium, so that the extra yolk can be delivered (Heming and Buddington 1988).

#### **3.4.7 Gross efficiency of growth**

This study demonstrates that *H. portusjacksoni* is highly efficient at converting yolk into body mass with GEG's ranging from 77-85% at 18, 20 and 22°C respectively. There were no significant temperature effects suggesting that this temperature range is within the normal developmental range encountered in this species. These values are directly comparable to the few other elasmobranch studies (Table 3.4) and are higher than other groups of animals. This illustrates that elasmobranchs are more efficient in converting yolk than other groups.

GROUP	temp.	GEG	source
<b>teleosts</b>			
<i>Salmo trutta</i>	10	70.0	Marr 1966
<i>Salmo iridens</i>	?	60.0	Wourms 1981
<i>Salmo gairdneri</i>	7	70.0	Escaffre and Bergot 1984
<i>Salmo gairdneri</i>	15	68.3	Rombough 1988a
<i>Paralichthys dentatus</i>	16	63.5	Johns and Howell 1980
<i>Paralichthys dentatus</i>	21	65.3	Johns and Howell 1980
<i>Clupea harengus</i>	?	71.0	Wourms 1981
<i>Gadus morhua</i>	5	84.0	Davenport and Lönning 1980
<i>Oncorhynchus tshawytscha</i>	6	67.9	Heming 1982
<i>Oncorhynchus tshawytscha</i>	8	63.0	Heming 1982
<i>Oncorhynchus tshawytscha</i>	10	60.5	Heming 1982
<i>Oncorhynchus tshawytscha</i>	12	48.8	Heming 1982
<i>Salvelinus alpinus</i>	4	68.9	Gruber and Weiser 1983
<i>Salvelinus alpinus</i>	8	69.5	Gruber and Weiser 1983
<i>Anchoa mitchilli</i>	26	76.0	Houde and Schekler 1983
<i>Archosargus rhomboidalis</i>	26	73.9	Houde and Schekler 1983
<i>Achirus lineatus</i>	28	77.5	Houde and Schekler 1983
<i>Hippoglossus hippoglossus</i>	6	77.9	Finn et al. 1991
<b>elasmobranchs</b>			
<i>Scyliorhinus caniculus</i>	?	79.0	Wourms 1981
<i>Scyliorhinus canicula</i>	15	83.4	Diez and Davenport 1987
<i>Heterodontus portusjacksoni</i>	18	77.7	this study
<i>Heterodontus portusjacksoni</i>	20	82.1	this study
<i>Heterodontus portusjacksoni</i>	22	85.3	this study
<b>reptiles</b>			
<i>Iguana iguana</i>	28	26.0	Ricklefs and Cullen 1973
<i>Emydura macquarii</i>	30	79.3	Thompson 1983
<i>Amphibolurus barbatus</i>		70.0	M. Packard et al. 1985
<i>Crocodylus johnstoni</i>		87.0	Whitehead 1987
<i>Crocodylus johnstoni</i>	29	56.3	Whitehead et al. 1992
<i>Crocodylus johnstoni</i>	31	59.4	Whitehead et al. 1992

Table 3.4 Gross efficiencies of growth in fish and oviparous vertebrate groups that rely on yolk reserves as the sole or main source of nutrition. GEG was calculated as the ratio of the average dry mass of yolk-free hatchling to the average predicted dry mass of yolk, multiplied by 100. Calculations for teleosts may or may not include yolk reserves.

A direct comparison of growth efficiencies between species requires examination of identical criteria such as environmental temperature regimes and stage of development (Blaxter 1969). Equivalent stages are necessary for any useful comparisons. In addition, the period within which data are taken must be considered in the analysis. That is, an equal time frame such as fertilisation to 90% yolk absorption or to complete yolk exhaustion. Blaxter (1969) demonstrated the variation caused by using different developmental stages in calculating GEG, with values ranging from 35-80%.

Gross growth efficiencies of oviparous fish have been calculated by many authors (see Table 3.4). Needham (1964) termed this as "gross" because not all the nutrients from the yolk are successfully converted into living tissue. It is less than a 100% transformation with energy from the yolk used for growth, respiration, embryonic activity and excretion. Nevertheless, it is a useful term for comparative purposes. It allows an estimate of the efficiency with which available nutrients can be utilised for growth under different conditions.

The results from other studies are reported (Table 3.4), but have little comparative value because of the discrepancies in data collection mentioned above. Only the data from oviparous species will be considered because viviparous fish embryos do not rely totally on yolk. In such embryos, the mother provides a continuous food supply, either by an maternal-foetal connection, provision of infertile eggs or edible siblings or an attached external yolk sac (Wourms 1977, 1981, Gilmore et al. 1983, Tanaka et al. 1990). Hence, from a small egg, the resulting neonate is large. If maternal sources are ignored, the calculated efficiency can be in excess of 12,000 % (in the Sand Tiger pup) (see Wourms 1981 Table 3).

While yolk absorption rates in teleosts increase with increasing temperature (Garside 1966, Santarre 1976, Hamor and Garside 1977, Howell 1980, Heming 1982) the efficiency with which yolk is converted to biomass may either decline due to increasing catabolic losses (Ryland and Nichols 1967, May 1974, Canino 1994) or be unaffected (Johns and Howell 1980, Quantz 1985). The literature reporting the effect of temperature on growth efficiencies is conflicting. Heming (1982) found a gradual increase in the efficiency of conversion in chinook salmon, *Oncorhynchus tshawytscha*, at lower temperatures. However, Gray (1928) claimed that decreased temperatures add to the cost of maintenance of development in trout, *S. trutta*, embryos and therefore

allow less yolk to be directed into embryonic development. Garside (1966) reported retarded growth at lower temperatures and hypoxia in embryonic trout. Later, Hamor and Garside (1977) found the same result in salmon embryos, *S. salar*. Hypoxia and increased temperature may fail to meet the oxygen demands of the embryo and retard growth. Hence the yolk is used less effectively for growth. However, Rombough (1988a) found no difference in the efficiency of growth between 6-15°C. Similarly, no significant difference was found between 18 and 22°C in this study.

Despite the paucity of data, the elasmobranch species appeared to be more efficient at transforming yolk into tissue than teleosts. The lower metabolism of elasmobranchs compared to teleosts (Brett and Blackburn 1978) may partially explain the greater proportion of energy allocated to growth in these species and the resultant higher efficiencies. However it is more probable that discrepancies in calculations, based on incomplete yolk absorption (for example Marr (1966), Heming (1982) and Rombough (1988a)), lead to an underestimation of growth efficiency when compared to calculations based on complete yolk absorption in *H. portusjacksoni*. Further problems arise when estimating teleost efficiencies because teleosts hatch from their eggs as free-swimming larvae with large internal yolk stores. Complete yolk utilisation occurs within a period of days but the efficiency post-hatch is greatly reduced because of the increase in activity. Overall teleost GEG are therefore likely to be underestimated.

### 3.5 SUMMARY

At constant incubation temperature, *H. portusjacksoni* development was absent at 10°C and significantly delayed and not completed at 15°C. At temperatures of 18, 20 and 22°C, development occurred successfully. This 4°C difference in incubation temperature increased incubation from 291-379 days or by 24%. At the lower temperatures, the rate of internal yolk utilisation was reduced. However, reduction of external yolk occurred at the same rate due to the combination of embryonic utilisation and formation of internal yolk stores. Despite the increase in incubation period at the lower temperatures, there was no significant difference in hatchling mass (wet or dry), length and internal yolk stores at the different temperatures.

This study reported several allometric relationships between the initial egg mass and egg components and hatchling mass. Predictably the initial egg mass is positively correlated with yolk, albumen and egg capsule masses, and the resultant hatchling mass. The progressive increase of embryo mass with length was also described which allowed the estimation of mass from total length, a particularly useful relationship in the very small embryos. Egg mass did not affect incubation period, with larger eggs producing larger embryos that consumed the greater yolk stores more quickly.

Growth patterns were described by logistic growth equations. Initial growth rates were similar at 18-22°C but lower temperatures extended the plateau phase. External yolk was depleted in a reverse sigmoidal manner to that of growth. Initially absorption was slow as little constructive embryonic growth occurred, followed by a marked increase in external yolk depletion. At a time when external yolk was minimal, internal yolk stores were maximal. The internal stores provided the necessary material for growth for the remainder of incubation. Hatching occurred when internal yolk was less than 1% of total embryonic mass.

The gross efficiency of growth ( $GEG = \text{dry mass of yolk-free embryo} / \text{dry mass of initial yolk} \times 100$ ) was high, ranging from 78-85% at 18, 20 and 22°C respectively. The effect of temperature on the GEG was not significant as hatchling masses (wet and dry) were similar at the three temperatures, despite a 90 day difference in incubation periods. This refuted the hypothesis that lower incubation temperatures which extended the incubation period, reduced the efficiency in which yolk was transformed into animal tissue.

## Chapter 4 ENERGETICS OF DEVELOPMENT

### 4.1 INTRODUCTION

Embryos developing from eggs which are laid externally are provided with all the energy necessary to complete development. Energy is supplied in most animal groups by an egg yolk; in others (for example birds), albumen may provide additional nutritional benefits (Ar et al. 1987, Whitehead 1987), although the contribution may be small. Fish eggs consist of a yolk surrounded by a peri-vitelline fluid and completely bound by a thin outer membrane. In oviparous elasmobranchs, the outer membrane is considerably thickened into a capsule and the peri-vitelline fluid (referred to as albumen), is replaced by seawater early in the incubation. In these embryos, the yolk is the sole energy store for all facets of growth.

There are many ways of examining the energetics of embryonic growth, of which I examine three ; energy budgets, cost of development and gross production efficiency.

#### 4.1.1 Energy budgets

Since the flow of energy in oviparous species takes place within a closed system the energetics of embryonic development is most simply described by energy budgets. The initial store of energy is set by the female parent when the egg is laid, and the efficiency with which the embryo utilises this stock is determined by several environmental factors, such as temperature, oxygen, light and salinity (Quantz 1985). I have chosen to examine the effect of temperature in this study. Conditions of oxygen, light and salinity were kept constant for the entire incubation period. Because the yolk provides a quantifiable amount of energy, a budget for its subsequent allocation in *H. portusjacksoni* can be made. The total energy in the system can be quantitatively distributed into the three major areas of growth, respiration and excretion:

$$\text{Total yolk energy} = G + R + E$$

where G = energy transferred for growth

R = energy utilised in respiration

E = energy lost in excretory products

A large proportion of the available energy is transferred into animal tissue (G). The respiratory component (R) incorporates the costs of synthesising the embryo (cost of growth) and the costs of maintaining the embryo in a steady state (cost of maintenance)

(Vleck and Vleck 1987, Konarzewski 1995). Maintenance is likely to be an important part of this component in long incubating species such as *H. portusjacksoni* because the embryonic tissue mass must be maintained for a considerable time. The final component of the budget is the energy lost in the waste products themselves (E). This component is likely to be small as found by Rombough (1988a) for steelhead embryos, *S. gairdneri*.

The initial amount of energy available in the fresh yolk can be measured by calorimetric analysis. The energy allocated towards growth can be measured as the energy content of the hatchling. The total energy consumed in respiration can be calculated by integrating the metabolic rate measurements over the incubation period and calculating the area underneath the curve (Vleck and Bucher 1998). Calorific equivalents (Brett and Groves 1979) for the oxygen consumed allow a direct estimation of the amount of energy utilised by respiratory mechanisms.

The respiratory component of the energy budget incorporates the cost of growth and the cost of maintenance, and the relative proportion of each can be assessed (Vleck and Vleck 1987). The progressive costs involved in respiration throughout incubation are determined directly from metabolic rate measurements (kJ/day), and the cost of producing an embryo (kJ/day) can be calculated from growth rates (g/day) and an energetic cost of growth (kJ/g). The cost of maintenance is calculated as the difference between the two. The qualitative importance of the energetic cost of maintenance to developing bird and reptile embryos has been analysed by many authors (Ricklefs and Cullen 1973, Ackerman 1981b, Hoyt 1987, Vleck and Vleck 1987, Whitehead 1987, Whitehead et al. 1992, Thompson and Stewart 1997), based on hatchling energetics. Ricklefs and Cullen (1973) suggested that increased incubation periods in poikilotherms, as a direct result of lowered temperatures, increase the maintenance component and consequently reduce the efficiency of energy utilised for growth. Ackerman (1981) and Vleck and Hoyt (1991) countered this by arguing that reduced temperatures effectively decrease the metabolic processes and hence reduce energy consumption. Thus, costs attributed to maintenance would not appear to increase with longer incubations. The embryos of *H. portusjacksoni* offer an opportunity to investigate the question of increased costs associated with increased incubation period. This study demonstrates a four degree reduction in incubation temperature significantly

increases the duration of incubation (Chapter 3, Table 3.1, p. 73). I measured the energetics of growth and respiration at each of the three experimental temperatures producing a budget, and determine the relevance of the incubation period on growth and the maintenance requirements (as determined by respiratory costs).

#### **4.1.2 Cost of development**

To date, the cost of development (that is, the amount of oxygen it takes to produce a given dry mass dry mg of hatchling) has been estimated only once for elasmobranch embryos (Diez and Davenport 1987). This study measures the costs in *H. portusjacksoni* and compares it to other vertebrate embryos.

#### **4.1.3 Gross Production Efficiency**

The changes in the energy content of each component of the egg, that is, the embryo and internal and external yolks, are examined throughout incubation to describe the energy dynamics of the system. The albumen, present for only a short time, is excluded from analysis. Dry masses and energy densities of each component are measured and used to determine how the energy of the system is distributed. The efficiency with which energy is transferred into animal tissue can be examined at different times throughout the incubation by measuring what I term a Progressive Efficiency (PE) or at the end of incubation by measuring the Gross Production Efficiency (GPE). These measure the percent of the initial available energy that has been transferred to the embryo or hatchling tissue mass. The GPE differs from the Gross Efficiency of Growth (GEG) (Chapter 3, Section 3.1.4, p. 63) because it deals with the transfer of energy rather than dry mass. The GPE has been investigated in birds (Vleck et al. 1980, Pettit et al. 1984, Bucher and Bartholomew 1984, Ar et al. 1987), reptiles (Rickleffs and Cullens 1973, Whitehead 1987, Whitehead et al. 1992), and teleost fish (Johns and Howell 1980, Eldridge et al. 1982, Gruber and Weiser 1983, Quantz 1985, Rombough 1988a, Finn et al. 1991, 1995). Inclusion of the efficiency in elasmobranchs will help to complete the evolutionary picture of energy transfer in embryos.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Energetics

#### 4.2.1.1 Embryo mass and percent water

Energy contents are expressed on a mass-specific basis, so egg and embryo components were weighed to 1 mg on a Sartorius 1265 MP balance. Embryos were sampled at selected intervals (stages 4-15) in conjunction with the staging and growth sampling (Chapter 2, Section 2.2.5, p. 17, Chapter 3, Section 3.2.2, p. 65). The top third of the broad end of the egg capsule was removed and the egg contents exposed. The embryo was killed by dissolving a lethal dose of anaesthetic tricaine methanesulphonate (Sandoz MS-222) to one litre of seawater (ca. 1g/g of embryo) and adding it to the fluid surrounding the embryo. The egg components (yolk, albumen and embryo) were then separated by gently pouring the contents into a large, glass petri dish (100×100×40 mm). All components were placed onto tared petri dishes and the wet masses recorded. The entire volume of albumen was collected with a plastic 20 ml syringe and weighed. The dead embryo was separated from the yolk sac by cutting the yolk stalk close to the embryo with scissors. The cut ends of the yolk stalk were pinched together to prevent yolk leakage from both the external yolk sac and internal yolk sac (if present). The embryo was weighed (whole mass) and then dissected to remove any internal yolk and reweighed (yolk-free mass). The internal yolk was then weighed. Finally the external yolk (contained within a yolk sac) was weighed. The dissection was performed quickly, taking only 1-2 min, thus keeping dehydration of the egg components to a minimum. To obtain dry masses of yolk and albumen, the samples were thoroughly mixed, and homogenous sub-samples of each were taken, weighed, dried overnight in an oven at 70°C, and reweighed until constant dry mass was achieved (defined as a loss of <0.1% per hour). This took approximately 24 h.

Percent water for yolk and albumen was calculated by the equation :

$$\% \text{ water} = \left( \frac{\text{wetmass} - \text{drymass}}{\text{wetmass}} \right) \times 100 \quad (\text{eq. 4.1})$$

To obtain dry mass of yolk-free embryos, the wet yolk-free embryo was diluted with exactly three times their mass of distilled water (weighed on a balance), placed in a blender and homogenised to create a smooth paste. A sub-sample was removed,

weighed and dried to a constant mass at 70°C. This was done because the embryo dried as a hard, plastic-like material which was impossible to grind with a mortar and pestle into a homogenous powder form for bomb calorimetry. The water added was taken into account in the calculation of dry mass of the embryo.

The percent water in the yolk-free embryo was calculated with the equation below, based on only one quarter of the total wet mass in the homogenised sample being yolk-free wet embryonic mass.

$$\% \text{ water} = \left( 1 - \frac{(4 \times \text{dry mass})}{(\text{total wet mass})} \right) \times 100 \quad (\text{eq. 4.2})$$

#### 4.2.1.2 Energy density

Dry samples of yolk, albumen and homogenised embryo were individually crushed into a homogenous powder with a mortar and pestle. Pellets were produced in a press and placed over silica gel into a desiccator. When ready to process, the pellets were weighed to the nearest 1.0 mg and placed into a tared metal cup inside a Gallencamp Ballistic Bomb Calorimeter. The pellet was burnt under an oxygen pressure of 25 atmospheres and the heat released recorded as a voltage output on a pen recorder. The mass of residual ash was recorded and subtracted from the initial sample mass. Using dry benzoic acid as a standard of known energetic density (26.455 kJ/g), the ash-free energy density of the sample was determined (kJ/g).

#### 4.2.2 Cost of development

The cost of development was calculated as the total amount of oxygen consumed throughout incubation divided by the dry mass of the yolk-free hatchling (in mmol O<sub>2</sub> g<sup>-1</sup> dry mass). Consumption rates (μmol.min<sup>-1</sup>) at 18, 20 and 22°C were measured for embryos from open capsules (stage 11) through to hatching (stage 15) (see section 5.2.1 p. 129) and plotted against incubation time. The total oxygen consumed was measured by calculating the area under the plot relating oxygen consumption and time. Oxygen consumption of embryos younger than stage 11 was considered to be negligible as the embryo prior to opening weighed less than 1g (Chapter 2, Table 2.4, p. 23) and the accumulative amount of oxygen consumed was small.

### 4.2.3 Production efficiency

The progressive efficiency (PE) throughout incubation was calculated as the percentage of energy converted into yolk-free embryo from the initial energy available in the fresh yolk taking into account the unused internal reserves.

$$PE = \frac{E(\text{y-f embryo})}{(E(\text{yolk}) - E(\text{unused yolk}))} \times 100 \quad (\text{eq. 4.3})$$

where E (y-f embryo) = the energy in the yolk-free embryo (kJ)

E (yolk) = the initial energy available in the yolk (kJ)

E (unused yolk) = the energy in the unused yolks (internal plus external) (kJ).

Similarly the Gross Production Efficiency (GPE) was calculated as the percentage of energy converted into yolk-free hatchling tissue from the initial energy available in the fresh yolk with the following equation.

$$GPE = \frac{E(\text{y-f hatchling})}{(E(\text{yolk}) - E(\text{unused yolk}))} \times 100 \quad (\text{eq. 4.4})$$

where E (y-f hatchling) = the energy in the yolk-free embryo (kJ)

E (yolk) = the initial energy available in the yolk (kJ)

E (unused yolk) = the energy in the unused yolks (internal plus external) (kJ).

### 4.3 RESULTS

#### 4.3.1 Relative water content in yolks and albumen

Results for relative water content in both external and internal yolk were variable and showed inconsistent patterns. In many cases there was no significant change in water content in external and internal yolk with changing yolk mass or with incubation time. At all temperatures, the mean water content for internal yolk was less than that of external yolk (Table 4.1). An ANOVA performed on data relating water content and temperature showed that temperature had no significant effect on the mean water content of either external or internal yolks. The samples of albumen were  $95.2 \pm 0.17\%$  water (n=4).

temp.	external yolk	internal yolk	ANOVA
18°C	43.2 ± 1.26 (6)	37.9 ± 0.90 (10)	F = 12.5 P<0.01
20°C	40.9 ± 0.42 (29)	38.3 ± 0.61 (13)	F = 12.6 P<0.01
22°C	41.1 ± 0.98 (5)	36.2 ± 0.52 (9)	F = 24.1 P<0.01
ANOVA	F = 2.37 P>0.05	F = 2.3 P>0.05	

Table 4.1 Mean relative water content (%) in external and internal yolks at 18, 20 and 22°C. Data are mean ± standard error (n). F statistics relate to ANOVA tests performed across and down the table.

#### 4.3.2 Relative water content in embryos

The water content in yolk-free embryos fell as the wet mass increased through incubation (Figure 4.1). Early embryos were 84-93% water, while hatchlings varied between 67-72% water (Table 4.2). A polynomial equation was fitted to the data (combined for all temperatures) :

$$\%W = -0.0001 M^3 + 0.0207 M^2 - 1.1046 M + 89.139 \quad n=41 \quad r^2=0.894 \quad (\text{eq. 4.5})$$

where %W = percent water and M = yolk-free embryonic wet mass (g).

Table 4.2 represents mean water content in hatchlings from the three temperatures. There were no significant differences in hatchling water content at the three experimental temperatures (F = 2.980, P>0.05).

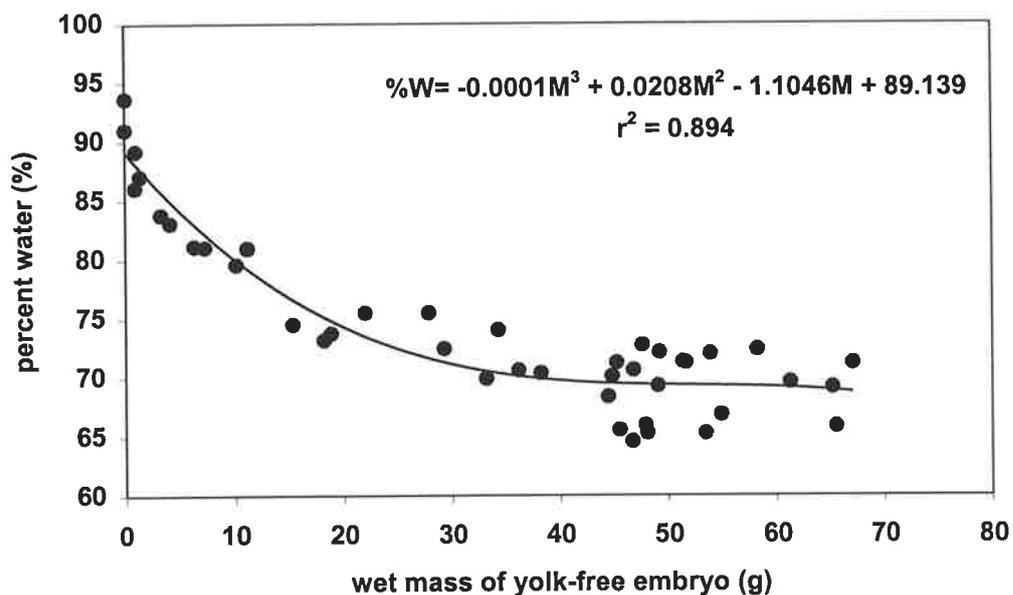


Figure 4.1 Relative water content (%) of yolk-free embryos at all temperatures.

Temp.	Wet mass (g)	Dry mass (g)	Percent water
18°C	56.85 ± 2.42 (14)	16.08 ± 0.88 (6)	71.7
20°C	58.79 ± 1.65 (17)	17.34 ± 0.44 (12)	70.6
22°C	54.91 ± 1.85 (17)	17.65 ± 0.81 (11)	67.9

Table 4.2 Mean hatchling water content ± SE (n).

### 4.3.3 Energy density of yolks and albumen

Energy density of external yolks (stages 4-13) at 20°C and 22°C did not vary significantly with time (Figure 4.2) ( $t = -1.056$  and  $2.103$ ) or temperature ( $F = 0.325$ ) at the 5% level, therefore data were combined for these temperatures. The overall mean energy density of external yolk was 27.03 kJ/dry g throughout incubation (Table 4.3). There were not enough data for external yolk density at 18°C to include in the statistical analysis. Statistical analysis on the internal yolk densities (stages 12-14) were not carried out due to the low number of data points.

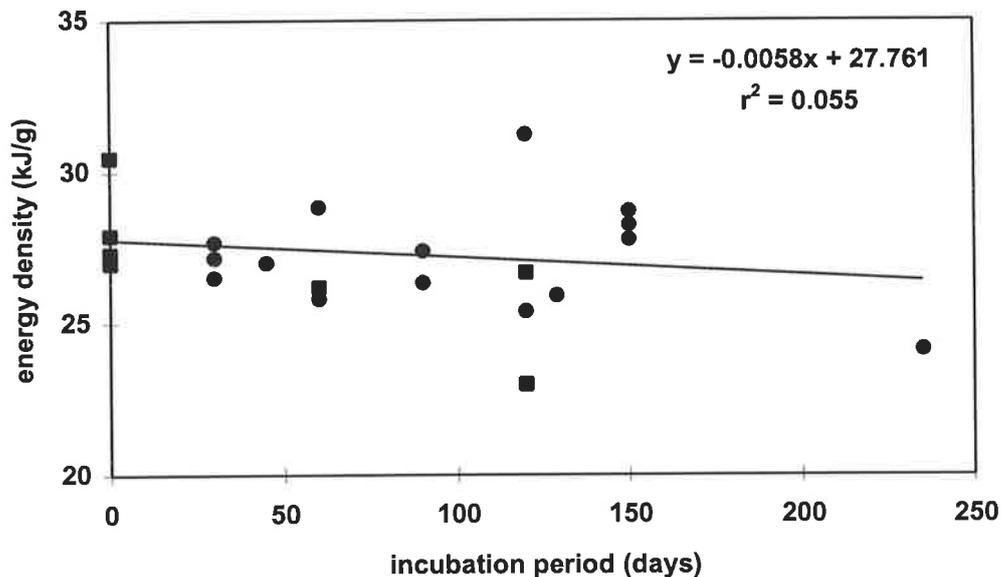


Figure 4.2 Changes in energy density of external yolks with time at 20°C (solid circles) and 22°C (solid squares).

Table 4.3 shows the energy densities of both internal and external yolk (kJ per dry g). Where statistics were possible, a mean, standard error and sample size are shown. Where sample size was less than 3 only a mean (n) is given.

temp.	Energy density (kJ/dry g)	
	external yolk	internal yolk
18°C	24.7 (2)	24.6 (2)
20°C	27.3 ± 0.35 (21)	28.9 ± 0.89 (3)
22°C	26.9 ± 0.65 (9)	24.4 (2)
overall	27.03	*

Table 4.3 Mean (± SE (n)) energy density of external and internal yolks taken from various embryo stages (4-15). \* indicates that no overall mean value was calculated and individual values for energy density for each temperature were used in calculations.

Dried samples of albumen did not burn in the bomb calorimeter or in a furnace at 600°C, and so did not allow determination of energy density.

#### 4.3.4 Energy density of embryos

In the first 45% of incubation, embryos less than 2 g (dry mass) had a lower mean energy density than those more than 2 g (dry mass) (17.54 and 27.46 kJ/g respectively). Embryonic energy density increases rapidly with dry mass until the embryo reaches approximately 2 g dry mass and then plateaus (Fig. 4.3). Data above 2 g yields insignificant changes of density with dry mass at the 5% level ( $F=5.98$ ). The regression line describing data above 2 g was as follows:

$$E = 27.99 - 0.185 M \quad n=14 \quad r^2=0.315 \quad (\text{eq. 4.6})$$

where  $E$  = energy density (kJ/dry mass (g)) and  $M$  = dry mass of yolk-free embryo (g).

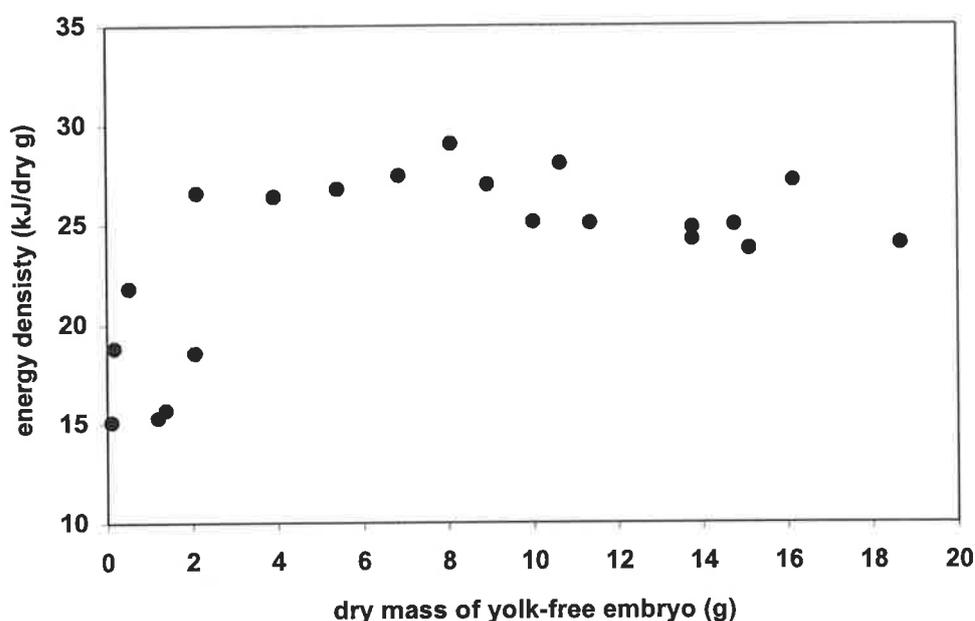


Figure 4.3 Energy density of yolk-free embryos (kJ/g) over time.

The total energy content of yolk-free embryos increased as incubation proceeded. Because incubation period varied significantly between temperatures, the relationship was plotted as the total energy content against the proportion of incubation at each temperature (Figure 4.4) and was described by the linear regression equations below:

$$18^{\circ}\text{C} \quad E(\text{tot}) = 6.637 \%I - 233.65 \quad n=8 \quad r^2=0.959 \quad T=10.2 \quad P<0.001 \quad (\text{eq. 4.7})$$

$$20^{\circ}\text{C} \quad E(\text{tot}) = 7.036 \%I - 321.34 \quad n=6 \quad r^2=0.984 \quad T=10.2 \quad P<0.001 \quad (\text{eq. 4.8})$$

$$22^{\circ}\text{C} \quad E(\text{tot}) = 8.634 \%I - 440.80 \quad n=7 \quad r^2=0.917 \quad T=10.2 \quad P<0.001 \quad (\text{eq. 4.9})$$

where  $E(\text{tot})$  = the total energy content (kJ/dry g) and  $\%I$  = the proportion of incubation (%).

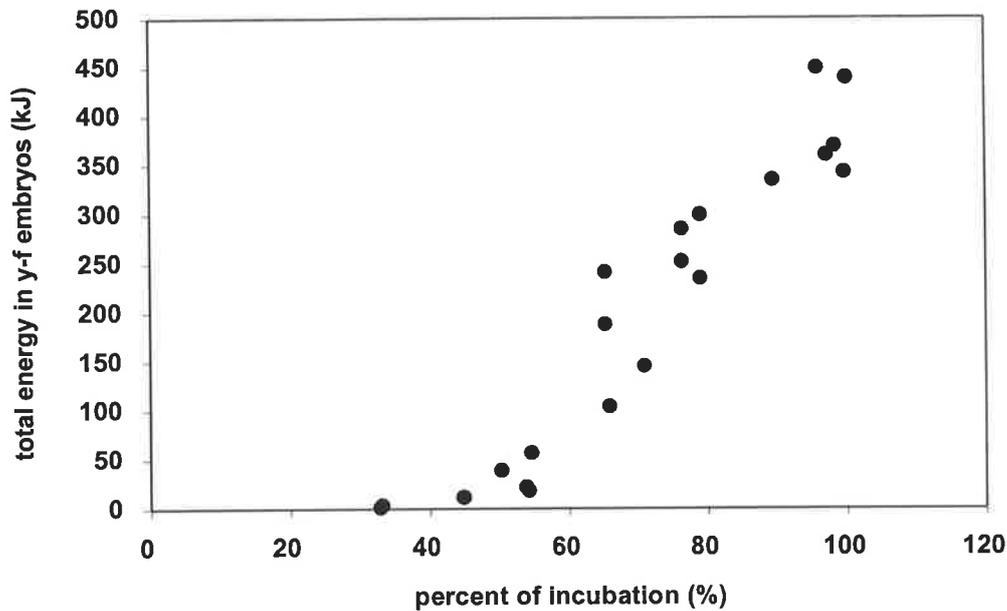


Figure 4.4 Total energy content (kJ) of yolk-free embryos against the proportion of incubation, at all temperatures.

#### 4.3.5 Energy budget

The energy budget for development in *H. portusjacksoni* embryos is given in Table 4.4. The energy allocated to growth and respiration accounted for 97-98.8% of that contained in the yolk at 20 and 22°C, while at 18°C the budget was exceeded by 4.3%. The total mean energy content and dry masses of initial yolk and hatchlings at 18, 20 and 22°C is reported in Table 4.6 (p. 112). Total oxygen consumed during incubation at 18, 20 and 22°C was 0.42, 0.29 and 0.27 mols O<sub>2</sub> (Fig. 4.5), translating to an energetic equivalent of 181.2, 123.8 and 116.0 kJ (assuming an oxy-calorific conversion of 19.382 × 22.4 kJ per mol O<sub>2</sub> consumed (Brett and Groves 1979)).

	Yolk energy (kJ)	=	Growth (kJ)	+	Respiration (kJ)	difference (kJ)	difference (%)
18°C	559.52	=	402.32	+	181.22	+24.02	+ 4.3 %
20°C	570.87	=	429.70	+	123.85	- 17.32	- 3.0 %
22°C	559.25	=	436.36	+	116.10	- 6.79	- 1.2 %

Table 4.4 The energy budget for *H. portusjacksoni* fitted well. Growth utilised 72-78% of the total available energy and respiration accounted for 21-32% of the total available energy.

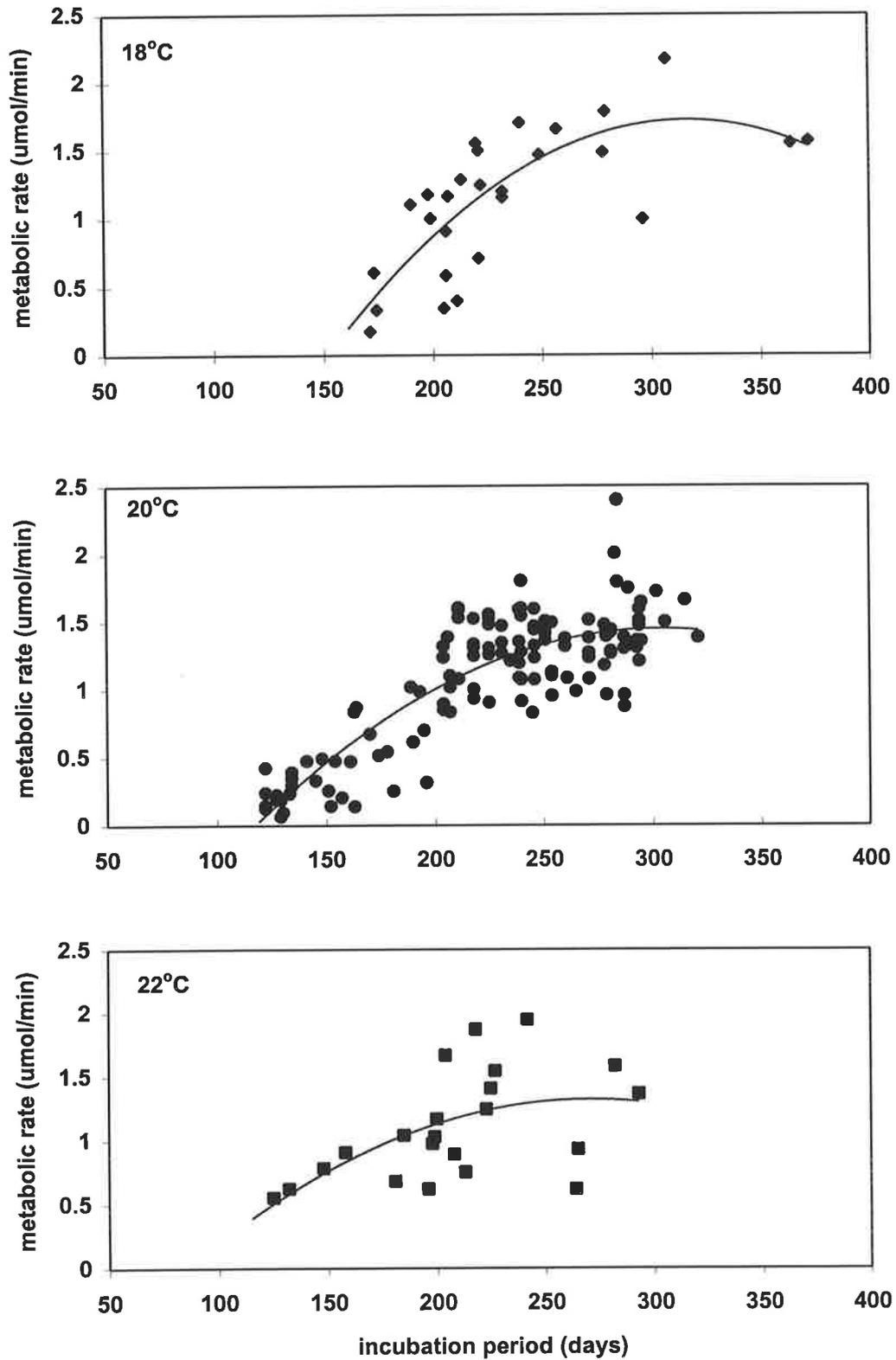


Figure 4.5 Total oxygen consumed after capsule opening at 18, 20 and 22°C.

#### 4.3.6 Energy cost of development

The mean metabolic cost of development for all temperatures was 19.17 mmol O<sub>2</sub> per dry g. Data for individual temperatures are shown in Table 4.5. The cost of development was similar at 20 and 22°C. Development at 18°C appeared to be the least efficient with the greatest costs and lowest hatchling mass. More oxygen was consumed over the significantly longer incubation at 18°C, consistent with greater maintenance costs, rather than reduced efficiency of energy transfer to the hatchling (from Table 4.6), although the trend would suggest that a reduction in efficiency did occur.

temp.	total O <sub>2</sub> consumed (mmol)	dry yolk-free hatchling mass (g)	metabolic cost of development (mmol O <sub>2</sub> g <sup>-1</sup> )
18°C	417	16.08	25.93
20°C	285	17.34	16.44
22°C	267	17.65	15.13

Table 4.5 The metabolic cost of development (mmol O<sub>2</sub> g<sup>-1</sup>) in *H. portusjacksoni* embryos at 18, 20 and 22°C.

#### 4.3.7 Energy allocation and gross production efficiency

Figure 4.6 relates energetic changes that occurred within the yolks and embryo during incubation. The energy content in the external yolk decreased as energy was transferred into embryonic tissue and later internalised as an internal sac. Internal stores of energy built up to a maximum at around the time the external yolk was depleted. These then became reduced as the embryo utilised them. By the end of incubation, almost all the energy in the system was present as embryonic tissue.

The progressive efficiency of energy transfer (PE) increased throughout the incubation as embryonic tissue mass accumulated (Figure 4.7). At approximately day 250 of incubation, PE decreased at all temperatures. This corresponds to when external yolk was absent and internal yolk was maximal and began to decrease in mass (Figure 3.5, p.74). For the majority of the incubation, the efficiency appeared to be greater the lower the temperature.

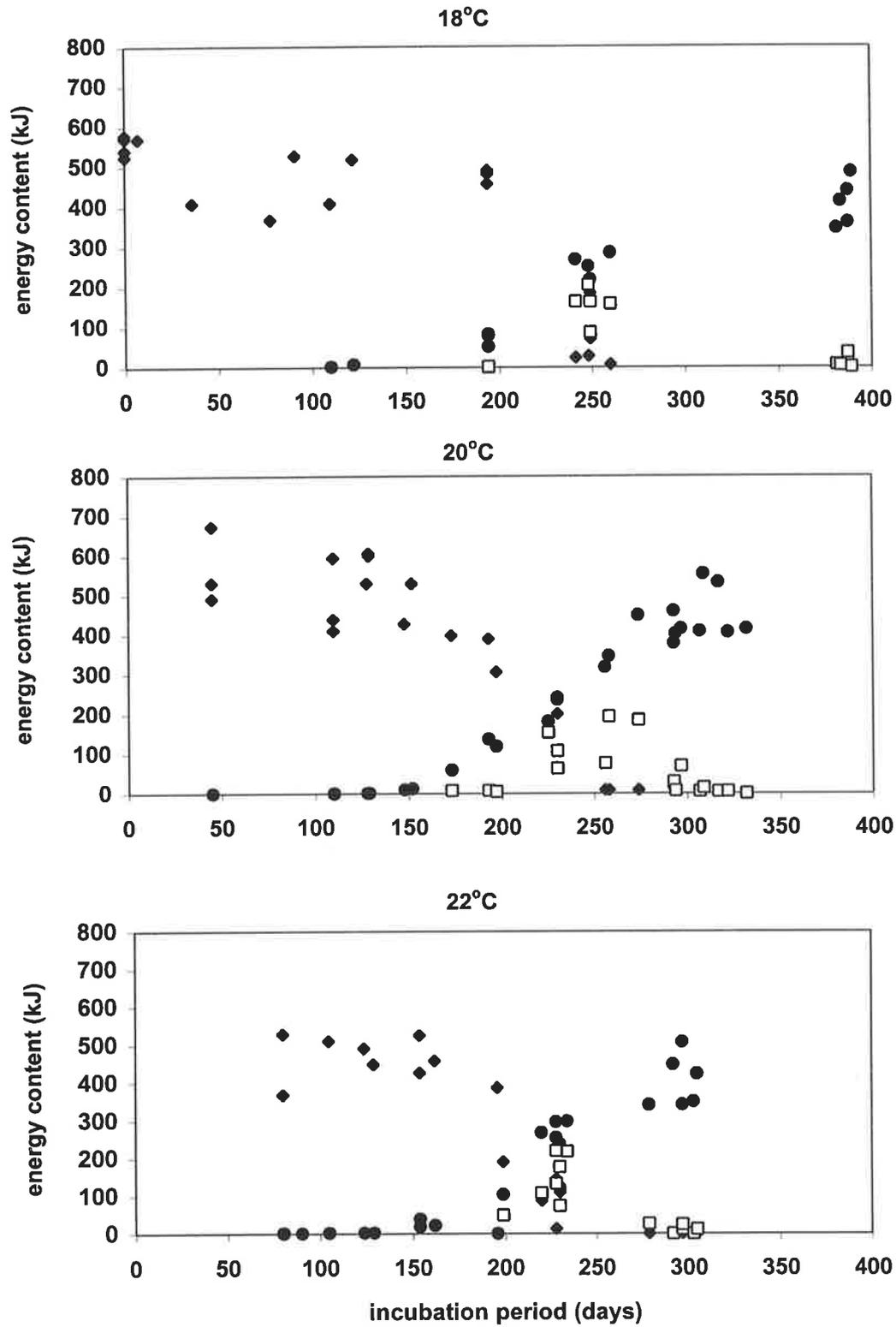


Figure 4.6 Changes in the energy content of yolk-free embryos (solid circles), external yolk (solid diamonds) and internal yolk (open squares) at 18, 20 and 22°C throughout incubation.

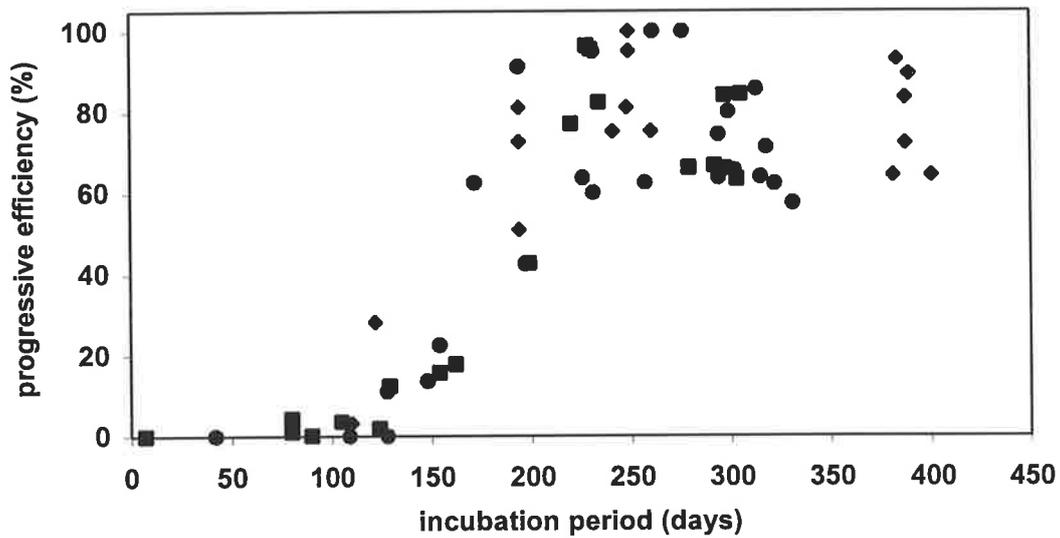


Figure 4.7 The progressive efficiency of energy as incubation proceeds at 18 (solid diamonds), 20 (solid circles) and 22°C (solid squares).

The efficiency of energy transfer from initial yolk to hatchling was calculated in a similar manner to which gross growth efficiency was estimated (Chapter 3, Section 3.2.6, p. 68). Using the data sources tabled, the efficiency of energetic conversion (=energy content of yolk-free hatchling divided by initial yolk energy content, multiplied by 100) was calculated for 18°, 20° and 22°C (Table 4.6). While a trend of increasing GPE with increasing temperature was found, it was not significant at the 5% level ( $t = 0.202, 1.364$  and  $1.015$ , for 18 vs 20, 20 vs 22 and 18 vs 22°C).

		<b>data source</b>	<b>18°C</b>	<b>20°C</b>	<b>22°C</b>
<b>Yolk</b>	mean dry mass (g)	Table 4.1	20.70	21.12	20.69
	mean energy density (kJdryg <sup>-1</sup> )	Table 4.3	27.03	27.03	27.03
	energy content (kJ)		<b>559.52</b>	<b>570.87</b>	<b>559.25</b>
<b>Hatchling</b>	mean dry mass (g)	Table 3.1	16.08	17.34	17.65
	mean energy density (kJdryg <sup>-1</sup> )	eq. 4.6	25.02	24.78	24.72
	energy content (kJ)		<b>402.32</b>	<b>429.70</b>	<b>436.39</b>
	<b>GPE (%)</b>		<b>71.9</b>	<b>75.3</b>	<b>78.0</b>

Table 4.6 Energy contents of initial yolk and yolk-free hatchlings at 18, 20 and 22°C. The gross production efficiency (GPE) is calculated as energy content of yolk-free hatchling divided by the energy content of the initial yolk multiplied by 100.

## 4.4 DISCUSSION

### 4.4.1 Energy budget

The energy budget constructed for *H. portusjacksoni* accounted for over 95% of the total amount of energy available in the initial yolk. A high proportion of energy is allocated to the construction of animal tissue (72-78%), while losses due to the respiratory component amounted to 22-32%. The unaccounted energy was excretory losses, which are shown to be small (less than 5%). The respiratory component was slightly lower than that reported for teleosts. During larval development of Atlantic halibut, *Hippoglossus hippoglossus*, 35% of the egg energy was dissipated in respiration (Finn et al. 1995), while Rombough (1988) found that steelhead embryos, *S. gairdneri*, consume a much higher 38-54% of energy for respiration but this included the more active post-hatch stages. Comparatively, many avian species (ranging from altricial to precocial developmental modes), use 20-40% of the total egg energy for respiration (Vleck et al. 1980, 1984, Ar et al. 1987). Rombough (1988a) constructed an energy budget for *S. gairdneri* from fertilisation through to 90% yolk absorption at temperatures ranging from 6-15°C. At 6-12°C, the budgets balanced well, with the energy consumed in the yolk being roughly equivalent (98.1%) to the energy transferred to the embryo plus the energy utilised in respiration, with little excretory loss. At 15°C, however, he found that the sum of the components was 12.5% greater than the total consumed, and concluded that 15°C was close to the upper thermal tolerance for the species and that the embryos may still have been stressed and more active while respirometry measurements were being taken.

### 4.4.2 Costs of maintenance and growth

Developing embryos must expend energy to maintain existing tissue and to synthesise new tissue. While growth continues, maintenance and synthesis costs must increase (Vleck and Hoyt 1991). When growth slows or ceases (i.e. during the plateau phase), maintenance of already synthesised material must continue. However, the contribution of maintenance costs to the total energy demands in poikilothermic embryos is still the subject of controversy. This controversy has been investigated in amphibians and many reptilian species, where a small alteration in incubation temperature has a substantial effect on the period of incubation, and in birds where there is a natural variability in the incubation period among the different species.

Pettit et al. (1984) suggested that the increases in the total oxygen consumed associated with longer incubation are reflections of greater maintenance costs, and result in a reduction of efficiency in seabird development. In contrast, other studies have artificially altered incubation periods in bird, reptilian and fish species and found that the incubation period does not affect the energy content of the resulting hatchling (Vleck et al. 1980, Hoyt 1987, Rombough 1988a, Whitehead and Seymour 1990, Whitehead et al. 1992, this study), although significantly more oxygen is consumed over the longer incubation (Ackerman 1981a, Seymour et al. 1991, Whitehead and Seymour 1990, this study). The efficiency of growth and the total amount of oxygen consumed over the incubation period (to 90% yolk utilisation) did not change in *S. gairdneri*, at 6-12°C despite over a 200% increase in the incubation period at the lower temperatures, suggesting that maintenance costs were unaffected (Rombough 1988a).

The data in this study support Ricklefs and Cullen (1973) hypothesis of increased maintenance costs associated with longer incubations at lower temperatures. As temperature was decreased and incubation period was lengthened, there was an increase in the energy utilised for respiration (Table 4.4, p. 107). In association with this, there was a small, but not significant, reduction in the energy efficiencies in *H. portusjacksoni* over the 4°C temperature range. Therefore the increase in respiratory costs suggests that the cost of maintenance must be increased at lower temperatures.

#### 4.4.3 Cost of development

The oxygen cost of developing one dry gram of *H. portusjacksoni* hatchling was 25.93, 16.44 and 15.13 mmol O<sub>2</sub> g<sup>-1</sup> at 18°, 20° and 22°C respectively. This suggests that at lower temperatures, where incubation period is significantly lengthened, the costs of development are greater. This is likely to be the result of increased proportion of maintenance to the energy consumed in respiration. The overall mean cost of development for *H. portusjacksoni* of 19.20 mmol O<sub>2</sub> g<sup>-1</sup> was similar to the 22.32 mmol O<sub>2</sub> g<sup>-1</sup> calculated from data for the dogfish embryo, *S. canicula* (Diez and Davenport 1987).

The effect of decreasing the incubation temperature was to increase the incubation period (Table 3.1, p.73) and hence increase costs of development as observed in higher respiratory and lower growth values (Table 4.4, p. 107). This effect is only obvious at the lowest temperature where a 4°C drop increases the incubation period by 30%,

resulting in an 8% reduction in hatchling energy and a 56% increase in the energy utilised in respiration.

Comparatively, the costs to produce one gram of dry hatchling has been evaluated in birds, reptiles and amphibians (Table 4.7). Embryonic birds are the most expensive to produce (34.42 mmol O<sub>2</sub> g<sup>-1</sup>), while reptilian and amphibian embryos appear to have lower but similar costs of 25.29 and 23.21 mmol O<sub>2</sub> g<sup>-1</sup> respectively. The mean energetic cost for elasmobranch development (20.98 mmol O<sub>2</sub> g<sup>-1</sup>) suggests similar costs among true poikilothermic embryos, and lower costs compared to the avian embryo. This table only reports a small sample of the studies for birds. However, the average cost of development in altricial and precocial birds is 33.99 mmol O<sub>2</sub> g<sup>-1</sup> (Vleck et al. 1984), close to the average calculated for the examples I have chosen to demonstrate comparison. Ackerman (1981a) and Vleck and Hoyt (1991) suggested that the difference in developmental costs between bird and reptilian data resided in the difference in incubating temperatures. Embryos that develop at higher temperatures have higher metabolic demands and hence greater oxygen consumption, but over a shorter incubation period, hence overall costs are similar. In contrast, this study showed that costs were markedly increased at 18°C where incubation period was significantly increased. Development at lower incubation temperatures is advantageous to the reptilian, elasmobranch and amphibian embryo with energetic costs 34-40% lower than the avian embryo for a comparable egg mass and incubation time (Ackerman 1981a).

#### 4.4.4 Gross production efficiency

The gross production efficiency of *H. portusjacksoni* embryos is greater than that reported for teleost fish, reptilian and avian embryos (Table 4.8). Increasing temperature had no significant effect on the efficiency with which energy was transferred into the final hatchling, although there was a slight trend for declining energy content at lower temperatures (Table 4.4, p. 107). Comparisons of energetic efficiencies between animal groups is difficult because of the differences in the energy sources available to their embryos. Some authors use the energy content of the yolk as the only energy available, when other components may be available as an energy source (e.g. albumen in birds, Ar et al. 1987). Whitehead (1987) collated data regarding the efficiencies of growth in several reptilian and avian embryos. He found that

group	species	cost of development	source	
Aves	<i>Pelecanus occidentalis</i>	29.91	Bartholomew & Goldstein 1984	
	<i>Puffinus pacificus</i>	33.04	Ackerman et al. 1980	
	<i>Gygis alba</i>	31.25	Pettit et al. 1981	
	<i>Pterodroma hypoleuca</i>	39.29	Pettit et al. 1982a	
	<i>Diomedea nigripes</i>	33.93	Pettit et al. 1982b	
	<i>Diomedea immutabilis</i>	32.59	Pettit et al. 1982b	
	<i>Anous stolidus</i>	39.29	Pettit et al. 1983	
	<i>Leipoa ocellata</i>	39.29	Vleck et al. 1984	
	<i>Alectura lathami</i>	30.50	Vleck et al. 1984	
	<i>Gallus gallus</i>	31.25	Davis & Ackerman 1987	
	<i>Anas platyrhynchos</i>	40.18	Hoyt & Rahn 1980	
			mean = 34.42	
	Reptiles	<i>Caretta caretta</i>	24.11	Ackerman 1981b
<i>Chelonia mydas</i>		19.64	Ackerman 1981b	
<i>Trionyx triungus</i>		21.88	Leshem et al. 1991	
<i>Dermochelys coriacea</i>		21.43	Thompson 1983	
<i>Emydura macquarii</i>		24.11	Thompson 1983	
<i>Emydura signata</i>		25.44 (24°C)	Booth 1998	
<i>Emydura signata</i>		27.65 (31°C)	Booth 1998	
<i>Natrix tessellata</i>		25.34	Dmi'el 1970	
<i>Vipera xanthina</i>		13.23	Dmi'el 1970	
<i>Alligator mississippiensis</i>		29.31	Thompson 1989	
<i>Crocodylus johnstoni</i>		28.57 (29°C)	Whitehead and Seymour 1990	
<i>Crocodylus johnstoni</i>		33.48 (31°C)	Whitehead and Seymour 1990	
<i>Eumeces fasciatus</i>		28.6	Thompson and Stewart 1997	
<i>Eumeces anthracinus</i>		31.3	Thompson and Stewart 1997	
			mean = 25.29	
Amphibians	<i>Pseudophryne bibroni</i>	23.21	Seymour 1999	
Elasmobranchs	<i>Scyliorhinus canicula</i>	22.32	Diez & Davenport 1987	
	<i>Heterodontus portusjacksoni</i>	19.20	this study	
		mean = 20.98		

Table 4.7 A comparative energetic cost of development among several animal groups. Units are mmol O<sub>2</sub> per dry g mass. In most cases, the cost of development was calculated from data available in the original paper. Where embryonic dry mass was not measured, it was calculated assuming a value of 82.1% water in hatchling reptiles (from unpublished data of Hoyt and Albers in Vleck and Hoyt 1991) or taken from other sources (e.g. for birds - Ar et al. (1987) and Rahn et al. (1984)).

Group	Species	GPE (%)	T (°C)	Source
Teleosts	<i>Paralichthys dentatus</i>	67.7	16-21	Johns & Howell 1980
	<i>Morone saxatilis</i>	43.8		Eldridge et al. 1982
	<i>Salvelinus alpinus</i>	68.9		Gruber & Weiser 1983
	<i>Scophthalmus maximus</i>	44		Quantz 1985
	<i>Salmo gairdneri</i>	59.3	15	Rombough 1988a
	<i>Hippoglossus hippoglossus</i>	59-61	6	Finn et al. 1995
	<i>Clarias gariepinus</i>	71		Kamler et al. 1994
Elasmobranchs	<i>Scyliorhinus canicula</i>	79		Amoroso 1960
	<i>Heterodontus portusjacksoni</i>	72	18	this study
	<i>Heterodontus portusjacksoni</i>	75	20	this study
	<i>Heterodontus portusjacksoni</i>	78	22	this study
Reptiles	<i>Iguana iguana</i>	45-52	28	Ricklefs & Cullen 1973
	<i>Crocodylus johnstoni</i>	73.9	29	Whitehead 1987
	<i>Crocodylus johnstoni</i>	71	29	Whitehead & Seymour 1990
	<i>Crocodylus johnstoni</i>	75	31	Whitehead & Seymour 1990
	<i>Crocodylus johnstoni</i>	65	29	Whitehead et al. 1992
	<i>Crocodylus johnstoni</i>	66.7	31	Whitehead et al. 1992
	<i>Crocodylus porosus</i>	65		Whitehead & Seymour 1990
	<i>Trionyx triunguis</i>	73		Leshem et al. 1991
Aves	<i>Agapornis roseicollis</i>	44.8		Bucher & Bartholomew 1984
	<i>Leipoa ocellata</i>	49.3		Vleck et al. 1984
	<i>Alectura lathami</i>	64.5		Vleck et al. 1984
	Review of 50 spp.	63.7		Ar et al. 1987

Table 4.8 Gross production efficiency of embryonic growth in several animal groups. Percent efficiency was calculated as the energy in yolk-free hatchling divided by the initial energy in yolk (minus unused yolks) multiplied by 100. Where experimental temperature is reported, it is shown.

conversions in reptiles based on yolk alone as the initial source were similar to those calculated on the basis of total egg energy content, thus suggesting that the yolk is providing the majority of the energy. In contrast, when the efficiencies based on the energy in the yolk alone were compared to efficiencies based on energy of the total egg in several avian embryos, the former produced extremely high efficiencies (128% in the chicken embryo) (Romanoff 1967). This suggests that other egg components make significant contributions to the energy budget of the developing avian embryo.

Whitehead (1987) reported general trends among the different groups which indicate similarity between reptilian, avian and teleost embryos in terms of the efficiency with which the initial egg energy is converted into hatchling tissue. Within the wide diversity of incubation period and degree of hatchling maturity in bird embryos, Ar et al. (1987) also produced similar energetic efficiencies among 50 species of birds. Comparisons of GPE between reptilian and avian embryonic development often consider the differences in the natural incubation temperatures and therefore incubation periods. Ricklefs and Cullen (1973) reported a lower GPE in the reptile, *Iguana iguana* (45-52%) compared to the chick embryo (63% - Brody 1945), claiming the extended incubation at the lower temperature created additional maintenance costs. Their data were based on efficiencies calculated on variable initial yolk contents and incompletely combusted hatchlings, thus it is likely that their calculations are underestimated. Later studies on reptilian development (Whitehead 1987, Whitehead et al. 1992) report efficiencies in the order of 65-74%, a figure exceeding that for general avian development (57% - Ar et al. 1987).

The variability among teleost fish data observed in Table 4.8 reflects the discrepancies in measurement criteria. A common difficulty arises because teleost embryos hatch as larvae with a substantial sized yolk from which they obtain energy for several days after hatching. During the period as a free swimming larvae, they are highly active and their oxygen consumption increases. Gruber and Weiser (1983) report a five-fold increase in oxygen consumption at hatching in Alpine Charr, *Salvelinus alpinus*, raised at 8°C. The additional requirements for activity in the free swimming larval stage reduce the energy available for growth, and result in lower efficiencies of growth. Some studies use hatchling energy contents to calculate the GPE (Gruber and Weiser 1983), while others measure energy contents of larvae at the time of 90% yolk absorption (Rombough

1988a) or complete yolk exhaustion (Quantz 1985), the latter two producing underestimates due to the increased metabolic demands of larval life. In addition, the larvae may begin exogenous feeding before the yolk is fully exhausted (Blaxter 1969), leading to underestimation of the energy efficiency of embryonic development.

When defining the quantity of energy successfully transformed into the final hatchling tissue mass, some studies refer to catabolism of embryonic somatic tissue that may occur at the end of incubation (Heming 1982, Quantz 1985, Wang et al. 1987, Heming and Buddington 1988, Finn et al. 1995). At this time, the energy needs of the embryo may be greater than the small remaining yolk can supply. The embryo resorts to catabolism for the necessary energy. Hence hatchling energy content may be less than maximum, leading to the underestimation of the efficiency of energy transfer (Heming 1982). The progressive efficiency of energy transfer in *H. portusjacksoni* began to increase around the time of capsule opening (Figure 4.7, p. 111). Diez and Davenport (1990) suggest that a gradual transition from anaerobic to aerobic metabolism in *S. canicula*, throughout development, due in part to the development of external gills and the opening of the capsule, increases the efficiency of energy transfer from yolk to embryo. Figure 4.7 shows that the progressive efficiency peaks prior to hatching, suggesting that some catabolism may occur close to hatching.

#### 4.4.5 Energy sources

The water content of *H. portusjacksoni* embryos decreased exponentially with time (Fig. 4.1, p. 104) and the pattern of energy density showed that energy density increased rapidly in embryos less than 2 g dry mass, as they absorbed more yolk solids and formed more body tissue (Fig. 4.3, p. 106). Up to this stage, the efficiency with which embryos transformed available yolk into body mass (Fig. 4.7, p. 111) showed that the embryos were still quite inefficient (<30%). However beyond 2 g (dry), the efficiency was high (> 60%) and fairly constant and the energy density of embryos did not change as incubation proceeded (Fig. 4.3, p. 106). Embryos larger than 2 dry g (stage 12 or 5 months) have completed a significant proportion of development and are morphologically very similar to the adult. They have large amounts of muscle and gut which was energetically more dense than skin or bone/cartilage (Meakins 1976), hence their overall density was greater than younger embryos (less than stage 12) which had less flesh. Similar changes in energy density were observed in the freshwater fish

examined by Meakins (1976). As the younger embryos develop, they accumulated more energetically rich substances into their bodies and increased their energy density.

#### 4.4.5.1 Yolk

The relationships between external and internal yolks through incubation were complex, with the initial external yolk mass being extremely variable (Chapter 2, Section 2.3.1, p. 19) and the mass of the internal yolk increasing mid-incubation and then decreasing mid-late incubation (Chapter 3, Fig. 3.5, p. 74). The general pattern of water usage in yolks of *H. portusjacksoni* showed no change over time or with mass, suggesting that water leaves the yolk at the same rate as yolk solids are absorbed. This is also seen in *S. canicula* where yolk water content was constant throughout development (Diez and Davenport 1990). This contrasts the situation found in bird and reptile eggs which must absorb water from the environment throughout the incubation in order to maintain egg humidity and to hatch successfully (Ar et al. 1987). Given that water and yolk solids are leaving the yolk at the same rate, it was not surprising to find the energy density of the yolks was unchanged throughout incubation.

#### 4.4.5.2 Albumen

In some groups of animals, such as birds, the albumen may provide an additional energy source to the developing embryo (Whitehead 1987), although it is limited because the proteins that make up the albumen are only partly oxidised and therefore can make only a part contribution (Ar et al. 1987). In addition to nutrients, the albumen also represents a substantial water store for the avian embryo, water being drawn from the albumen into the circulation and finally into the embryo itself. The jelly coats of amphibians such as the Korean fire-bellied toad, *Bombina orientalis*, have a significant role in the transport of amino acids from the media to the embryo (Cameron et al. 1986). Denaturation of the jelly coats reduces the ability of the coats to transport amino acids to de-jellied embryos. Eddy (1974) suggested that the relatively high proportion (25%) of protein in fish peri-vitelline fluid (= albumen) may infer a nutritional function. However, Heming and Buddington (1988) believe that the uptake of exogenous nutrients in oviparous embryos has little nutritional benefit because of low natural concentrations and slow transfer rates. The latter argument may be applicable to teleost embryos where the outer capsule (equivalent to the shell) creates a physical barrier to nutrient movements and the external media is freshwater or seawater which may be nutrient poor.

The inability to burn albumen samples in this study suggests a large inorganic content, thus arguing against a nutritive function for this species. In addition, the albumen is only present for a relatively short period (3-4 months) and not the entire incubation as it is in reptilian and avian eggs. The probability of a nutritive function of the albumen for *H. portusjacksoni* is further reduced because while the albumen is present in the egg, there is little overall embryonic growth (< 2% of the total embryonic mass is attained while the capsule is sealed).

#### 4.5 SUMMARY

The yolk provides all of the energy for growth and development in *H. portusjacksoni* eggs. The energy available is allocated to several functions, defined as growth, respiration and excretion. A budget was constructed calculating the relative contribution of growth and respiration. Excretion was ignored as a minor contributor. The budget accounted for up to 95% of the available energy at all temperatures. The majority of the energy was transformed into animal tissue (gross production efficiencies (GPE) of 71.9, 75.3 and 78% at 18, 20 and 22°C respectively), while respiration accounted for the remaining energy (32.4, 21.7 and 20.8% at 18, 20 and 22 °C respectively). The growth component was similar to other poikilothermic groups but higher than for birds, while the respiratory component was comparable among all animal groups. There was no significant difference in the GPE at the three temperatures, despite a 24% in the incubation period at the lower temperature. However, there was a general trend of decreasing hatchling mass and lower GPE as temperature decreased. The total oxygen consumed was similar at 20°C and 22°C but was markedly higher at 18°C. This produced similar developmental costs at 20°C and 22°C of 16.4 and 15.1 mol O<sub>2</sub> per g dry embryonic mass and greater costs at 18°C of 25.9 mol/g. The greater oxygen consumption and the smaller hatchlings at 18°C infer some additional maintenance costs at the lower temperature. The unit cost of producing one g of dry tissue mass was low in *H. portusjacksoni*, compared to avian, reptilian and amphibian development largely due to the lower basal metabolism of elasmobranchs.

## Chapter 5 EMBRYONIC RESPIRATION AND VENTILATION

### 5.1 INTRODUCTION

The primary respiratory organ in fishes are the gills. Respiration can also occur across the skin and yolk surfaces, depending upon the species and the stage of development (Kirshch and Nonnotte 1977, Lomholt and Johansen 1979, Feder and Burggren 1985, Steffensen and Lomholt 1985, Rombough 1989, 1998 and Takeda 1990). In some species (e.g. salmonids), cutaneous respiration accounts for 80% of the oxygen uptake (Rombough and Ure 1991, Wells and Pinder 1996b). The gills are located in the branchial chambers at the anterior end of the gut. Water is channelled through the gills by ventilatory movements, and respiratory gases ( $O_2$  and  $CO_2$ ) are exchanged between the water and blood in a countercurrent fashion (Grigg 1970). The gills are also an important site for heat and osmotic regulation (Carey et al. 1981, Bone and Marshall 1982), therefore ventilation through the gills must be carefully controlled to balance respiratory, osmotic and heat requirements.

There has been a great deal of research on the metabolism of teleost fish, in particular, adult and juvenile fish. Many of the studies on adults examine the effect of changes in environmental parameters such as oxygen and temperature on metabolism (Caulton 1977, Hughes et al. 1983, Nordlie et al. 1991, Petersen and Petersen 1990, Lezama and Günther 1992). The daily and seasonal fluctuations of these parameters stimulates research to comprehend the mechanisms behind survival in physiologically challenging conditions. For example, tropical fish species such as cichlids are often exposed to high temperatures and environmental hypoxia (Fernandes and Rantin 1989). Similarly the burrowing fish, *Ammodytes hexapterus*, is frequently exposed to low oxygen (Quinn and Schneider 1991). As a consequence these species are well adapted to such conditions by being able to physiologically regulate their oxygen consumption rates at very low ambient oxygen conditions.

There has been considerably less attention focused on metabolism in embryonic fish. Some metabolic investigations have been performed on teleost eggs, providing a better understanding of the changing energy requirements during development (Houde and Schekter 1983, Dabrowski et al. 1984, Rombough 1988a, Finn et al. 1995).

Investigations of embryonic teleost metabolism have been largely motivated by aquaculture activities, to maximise embryonic survival and therefore economical benefit

(Jones 1972, Divakarumi and Sharma 1990). More specifically, the effect of physical parameters ( $PO_2$  and pH) and developmental stage on metabolism of teleost eggs and larvae have been assessed by Spoor (1977, 1984), Davenport and Lönning (1980), Carrick (1981), Dabrowski et al. (1984) and Walsh and Lund (1989).

My study aims to investigate respiration in the *H. portusjacksoni* embryo throughout incubation. Because invasion of a sealed egg capsule is lethal, the hypothesis that the embryo is aerobic during the 4 months is investigated using the parameters of albumen  $PO_2$  and external morphology of the embryo. In particular I examine the possible role of the external gill filaments as respiratory aids. In addition, the diffusive capabilities of the capsule are calculated in order to determine if oxygen can diffuse in at a sufficient rate to maintain aerobic respiration or if there is enough oxygen in the egg at the time of laying to support the embryo for the first 4 months. The facility of oxygen movement through a given material is defined by Krogh's diffusion coefficient ( $KO_2$ ) (Dejours 1975). This parameter has been calculated for the capsules of two elasmobranch species (Diez and Davenport 1987, A. Gannon 1992) with varying results. The addition of a third species with much larger eggs will enhance the understanding of metabolism in the early stages of development. Oxygen consumption in embryos from open capsules (stage 11 onwards) is examined using both open and closed respiratory techniques, and the effects of mass, temperature and hypoxia are assessed.

### 5.1.1 Allometry of respiration

The oxygen consumption of an organism increases with body size, although not in direct proportion. The allometric relationship between oxygen consumption and body mass is a power function. Mathematically, the relationship is expressed as

$$\dot{V}O_2 = a M^b$$

where  $\dot{V}O_2$  is the rate of oxygen consumption,  $M$  is the body mass of the animal, "a" is the mass coefficient and "b" is the mass exponent (Winberg 1956). Values for "a" and "b" are derived from the intercept and slope of a log transformed relationship.

Allometric laws relating oxygen consumption and body mass in adult fish are often extrapolated to include larval and embryonic forms. This allometry may not hold true for early life stages because of the dynamics of development and growth in such stages. While the generalised mass exponent for fish is 0.8 (Winberg 1956, Fry 1957, Schmidt-

Nielsen 1984), larval studies demonstrate exponents closer to 1.0, indicating an isometric relationship between oxygen consumption and body mass (Weiser 1985, Finn et al. 1995). The availability of teleost eggs in large quantities has meant that this relationship has been well developed in this group through all stages, and it is also well established in adult elasmobranchs. However it is only in the last twelve years or so that studies on oxygen consumption in embryonic elasmobranch embryos have emerged (Diez and Davenport 1987, Arnold 1989, A. Gannon 1992, Tullis and Peterson 1997, Meehan et al. 1997), allowing the ontogenic relationship between oxygen consumption and body mass to be examined. The present study adds to the increasing data being gathered for elasmobranchs. The lack of comparable data for embryonic elasmobranchs is likely to be related to the reproductive strategy of viviparity which most elasmobranchs exhibit. The difficulties in maintaining live embryos outside the female's body make metabolic measurements difficult. Oviparous elasmobranchs reduce such problems because the embryo is easily accessible and metabolism can be measured directly as incubation proceeds.

### **5.1.2 Effect of temperature**

The positive effect of temperature on oxygen consumption has been well established in ectothermic animals. Throughout incubation *H. portusjacksoni* naturally encounters large variations in temperature (12-22°C)(Appendix 1, Fig. 7, p. 187), creating continuous changes in the physiological demands of the developing embryo. The effect of temperature on oxygen consumption can be described by  $Q_{10}$  values, that is, the ratio of oxygen consumption at temperature (t+10°C) over oxygen consumption at temperature t°C (Dejours 1975).

### **5.1.3 Effect of ambient oxygen**

Oxygen consumption can also be altered by environmental oxygen levels. When exposed to hypoxic conditions, adult and juvenile fish have the ability to either adapt physiologically, and/or exhibit avoidance behaviour, in an effort to remove themselves from unfavourable conditions. The sand goby, *Pomatoschistus minutus*, responds to gradual decreases in ambient oxygen by increasing its ventilatory response, and in severe hypoxic conditions, it actively avoids such conditions by swimming to the water surface (Petersen and Petersen 1990). Embryonic fish do not have the luxury of avoidance behaviour. The eggs are often fixed to a structure (weed or rocks) and cannot

be moved under normal circumstances. Fish embryos must therefore be more tolerant of adverse conditions if they are to survive periods of unfavourable conditions. Several studies done on fish embryos have shown that earlier stages (pre- free swimming) are more tolerant of hypoxic exposure. Embryonic smallmouth (*Micropterus dolomieu*) and largemouth bass (*M. salmoides*) are highly resistant to severe hypoxia (20 Torr) (Spoor 1977,1984). After hatch, they become increasingly more sensitive and survival is reduced, such that 10 day old larvae exposed to 20 Torr die. The tolerance to low oxygen may change with increasing embryonic or larval age (DeSilva and Tytler 1973). Early stages of herring and plaice embryos show some ability to regulate their oxygen consumption, but as they develop further, this ability is reduced. Later again, after metamorphosis when the respiratory pigment appears, larvae have an increased ability to regulate their oxygen consumption. Comparative elasmobranch data for adults and embryos are rare. Oxygen uptake decreases markedly in adult dogfish, *Scyliorhinus stellaris*, exposed to hypoxia (Piiper et al. 1970), while embryonic dogfish, *S. canicula*, survive extended exposure to anoxia by resorting to anaerobic metabolism (Diez and Davenport 1987). While naturally occurring environmental levels of oxygen remain high throughout incubation of *H. portusjacksoni* (Appendix 1, section 3.3, p. 188), internal oxygen levels of the capsule may fall to hypoxic levels, brought about by inadequate ventilation of the capsule by the embryo itself. This study aims to increase the knowledge regarding hypoxic effects on metabolism in embryonic elasmobranchs.

#### **5.1.4 Ventilation**

The flow of water through the elasmobranch gill is maintained by differential movements of the musculature of the orobranchial and parabranchial cavities and the opening of the external gill coverings. Water is drawn in through the mouth and spiracles and is expelled through the external gill openings (Butler and Metcalfe 1988, Graham et al. 1990). Water entering the spiracle may bypass the mouth, allowing simultaneous feeding and breathing. This is common in skates and rays where the gills are located ventrally and the spiracular openings are on the dorsal surface, and in bottom-dwelling sharks such as *H. portusjacksoni*, where spiracular inhalation prevents food particles clogging the gills during feeding. Water can also enter the first gill slit and subsequently serve the remaining gills (Fry 1957, Grigg 1970). Periodic reversal of water flow may also occur during prey capture and transport as observed in the swell

shark, *Cephaloscyllium ventriosum*, such that water enters and leaves via the mouth (Ferry-Graham 1999).

Several studies have primarily demonstrated new techniques to measure gill ventilation (Lutz 1930, Satchell 1959, 1960, Millen et al. 1966). However, in many other investigations, the physiological components of respiration and ventilation were assessed in relation to pollution effects and environmental hypoxia (Watters and Smith 1973, Burton 1979, Kerstens et al. 1979, Campagna and Cech 1981). The increase in water temperature associated with effluent outfall increases the respiratory and ventilatory work of fishes (Watters and Smith 1973, Burton 1979, Steffenson et al. 1984). Natural phenomena such as eutrophication of lakes reduce the available oxygen, causing an increase in the ventilation rate of *Orthodon microlepidotus* (Campagna and Cech 1981). Tropical fish species such as *Oreochromis niloticus* experience environmental hypoxia as the temperature of the water increases. This produces an increase in gill ventilation, resulting from an increase in breath volume rather than ventilation frequency (Fernandes and Rantin 1989).

Other studies examined the relationship between gill ventilation and acid-base regulation in elasmobranchs, because of the unusual nature of the elasmobranch blood (Randall et al. 1976, Heisler et al. 1988, Graham et al. 1990). Elasmobranchs have no Root shift and a weak Haldane effect compared to teleost fish (Lenfant and Johansen 1966, Wells and Weber 1983, Butler and Metcalfe 1989). The reported Bohr effects in elasmobranchs are extremely variable, with no effect or only a small Bohr shift in the sharks, *Squalus suckleyi* and *H. portusjacksoni* (Lenfant and Johansen 1966, Nash et al. 1976), and the skate, *Raja ocellata* (Graham et al. 1990), to very large effects in the sting-ray, *Dasyatis sabina* (Mumm et al. 1978). Ventilation appears to be matched with the regulation of acid-base parameters rather than oxygen requirements under conditions of reduced oxygen-related drive in the shark, *Scyliorhinus stellaris* (Heisler et al. 1988).

The investigations described above were all performed on juvenile or adult fish. Comparative embryonic studies are rare. The oviparous embryo is primarily independent of the surrounding environment, however it must largely rely on the external environment for its water, inorganic material and oxygen (Amoroso 1960). The potentially large respiratory barriers created in teleost egg masses, has prompted

studies examining the convective forces that dictate the flow of water around and within these egg masses (O'Brien et al. 1978, Davenport 1983, Peterson and Martin-Robichaud 1983). Only recently has there been an increased interest in ventilation through the egg capsules of oviparous skates (Long and Koob 1997) and sharks (Thomason et al. 1996, Meehan et al. 1997, Tullis and Peterson 1997). The pressure changes occurring in the capsule during tail pumping in little skates, *R. erinacae*, were measured and the ontogenetic changes in ventilation patterns were observed in the spotted dogfish, *S. canicula*, swell shark, *C. ventriosum*, and the bamboo shark embryo, *C. plagiosum*.

The oviparous elasmobranch embryo is confined to an anchored egg capsule for the duration of its incubation. Once fixed in position, the capsule is unlikely to be moved - to do so would be perilous to the survival of the embryo within (for example, egg capsules freed from their anchorage in storms are often washed up on the beach and the embryos perish). During its incubation, the embryo may be exposed to a variety of environmental conditions, such as temperature changes (particularly if located on a shallow reef) and hypoxia. It is important, therefore, to examine the respiratory and ventilatory responses of the developing embryo to potential environmental variables.

My study attempts to describe ventilation through the egg capsule of *H. portusjacksoni* in order to understand the changing needs of the embryo throughout incubation. I examine ventilatory frequency at three experimental temperatures to determine if changes in environmental temperature induces a ventilatory response. In addition, I investigate the ventilatory response to hypoxia, a condition the embryo might be exposed to at some time during the long incubation period. Finally, I attempt to correlate the embryos' respiratory and ventilatory requirements at various stages of development.

## 5.2 MATERIALS AND METHODS

### 5.2.1 Oxygen consumption rate in embryos from open capsules

It is generally accepted that the measurement of oxygen consumption under controlled, defined conditions is a reliable estimate of the metabolic rate of an animal. It assumes that the energy produced is by aerobic processes and that anaerobic pathways play a minor (if any) role. Controlled conditions are important for a reliable measurement of the rate of oxygen consumption because the rate is affected by many intrinsic and extrinsic factors. It is difficult to compare metabolic studies because not all factors are controlled in the same way. Important factors include the size of animal, acclimation to the experimental design, temperature, salinity, the state of nutrition, sex, degree of activity (diurnal or nocturnal), reproductive status, and the reliability and limits of the equipment used (Zeuthren 1953; Pritchard et al. 1958; Taylor 1976; Chan and Wong 1978, Morris and North 1984, De Silva et al. 1986, Divakaruni and Sharma 1990, Petersen and Petersen 1990, Nordlie et al. 1991, Oikawa et al. 1991).

There are two main methods to measure oxygen consumption in aquatic organisms; closed and open respirometry. Closed respirometry involves the placement of an animal in a chamber filled with air-saturated water, sealing it and monitoring the progressive decrease in oxygen tension with time. Problems may arise in these conditions from the accumulation of excretory products and the progressive hypoxia the animal induces, but Rombough (1988a) has shown that the build up of excretory products is not a problem. Open systems have overcome waste product build up by constantly circulating fresh water through the chamber. Knowing the flow rate of water through the chamber, the oxygen capacitance of water at the experimental temperature and the difference in  $PO_2$  in the inflow and outflow water, oxygen consumption can be calculated. However, open respirometry has inherent problems also. The flow must be sufficient enough to prevent the animal experiencing hypoxia, but allow a significant readable difference in the oxygen levels between the in- and outflow. Ideally, the outflow oxygen pressure should be kept to 10-20% below saturation to ensure normoxic conditions (Caulton 1978, Hughes et al. 1983, Eccles 1985, McCarthy 2000). In addition, the flow meter should be placed before the inflow channel to prevent any blockages (Hughes et al. 1983), so that accurate flow readings can be made.

The advantages and disadvantages of both closed and open respirometry must be evaluated relative to the experimental aims. When working with juvenile or adult fish, open respirometry appears to be preferred (Watters and Smith 1973, Kerstens et al. 1979, Hughes et al. 1983, Kaushik et al. 1984, Heisler et al. 1988, Bushnell et al. 1989, McCarthy 2000), because the oxygen consumption rates are likely to be high, and there are difficulties in providing a closed respirometer large enough to prevent rapid oxygen depletion. Closed respirometry is advantageous if the animal is small, that is, for eggs, larvae or small fish (Czihak et al. 1979, Carrick 1981, Degani and Lee-Galagher 1985, Divakaruni and Sharma 1990, Rønnstad et al. 1994, Marsh and Manahan 1999) where oxygen consumption is low and excretion rates negligible. There are exceptions where closed respirometry has been used for large fish (Scharold et al. 1989, Nordlie et al. 1991) and open systems have measured metabolism in eggs and larvae (Spoor 1977, 1984, Dabrowski et al. 1984). Some workers have employed both systems to assess metabolism in the same species and found no significant difference between the two (Rombough 1988a, Oikawa et al. 1991).

The rate of oxygen consumption in this study was evaluated with both closed and open respirometry. Measurements were taken from embryos (stages 11-15) at 15, 18, 20 and 22°C. The oxygen consumption of embryos incubated at 15°C was measured until stage 11 because freshly collected eggs incubated at this temperature did not develop past stage 11 and eventually died. To obtain further metabolic data for 15°C, it was necessary to collect older eggs from the field (2-3 months old) and complete the incubation in the laboratory at 15°C. After an equilibration period of a month in the laboratory, the oxygen consumption of these individuals was then measured until hatch.

#### **5.2.1.1 Measurement of the partial pressure of oxygen**

The partial pressure of oxygen ( $PO_2$ ) was determined by a thermostat controlled Radiometer (E5046) oxygen electrode and a Radiometer Blood Micro System (BMS2 MK3). The BMS was calibrated with a zero  $PO_2$  solution (sodium sulphite-sodium tetraborate solution) (Tucker 1967) and an air saturated solution of seawater. The zero solution was made fresh daily and injected into the BMS for calibration. The solution was left for 5 min. The BMS was then rinsed thoroughly with 10 ml of distilled water. A solution of air saturated seawater was then injected into the system in order to set the upper range (saturation). Saturation was calculated from the equation:

$$PO_2 = (Pb - P_{H_2O}) \times 0.2095 \quad (\text{eq. 5.1})$$

where  $PO_2$  is the oxygen partial pressure (Torr)

$Pb$  is the barometric pressure (Torr)

$P_{H_2O}$  is the water vapour pressure (Torr)

and 0.2095 is the fraction of oxygen in dry atmospheric air.

### 5.2.1.2 Closed respirometry

Closed respirometers were constructed from cylindrical polycarbonate plastic containers. The size of respirometer varied according to embryo size from 1 to 3 L, such that the available oxygen was not used up too quickly. Respirometers were placed in aerated aquaria and filled with seawater. The embryo was removed from its egg capsule and placed into a chamber, together with an air stone which aerated and circulated the water. The lid was placed loosely over the top while acclimatisation occurred. After 2-3 h, the air stone was removed, excess bubbles carefully removed from the surface of the chamber, and the lid positioned firmly to seal the respirometer. The chambers were taken from the aquarium and covered with black plastic so visual stresses were eliminated. Control respirometers (with no embryo in the chamber) were run at the same time. These were filled with aerated water and sealed immediately with no acclimation time. The experiment was run in constant temperature rooms in which air-conditioners maintained water temperature at the desired level ( $\pm 1^\circ\text{C}$ ).

A three-way stopcock inserted near the bottom of the chamber wall allowed sampling of the water over time. Samples were taken at regular time intervals and continued over 3-6 h until the  $PO_2$  was between 80-100 Torr.

Sampling was achieved by filling a 2 ml syringe with seawater of oxygen pressure approximating the oxygen pressure in the chamber at the time, and injecting into the chamber. The volume increase was possible as the lid was flexible. The syringe was emptied and filled at least three times to mix the water before a 2 ml sample was removed. The sample was immediately analysed for  $PO_2$ . Mass-specific oxygen consumption was calculated from equation 5.2. Background oxygen consumption of the seawater calculated from the controls was subtracted to calculate the consumption due to the embryo alone.

$$\dot{V}O_2 = [(((PO_2 i - PO_2 f) \times \beta \times V) / (M \times t))] \quad (\text{eq. 5.2})$$

where  $\dot{V}O_2$  = oxygen consumption ( $\mu\text{mol min}^{-1} \text{g}^{-1}$ )

$PO_2 i$  = initial oxygen pressure in the chamber (Torr)

$PO_2 f$  = final oxygen pressure in the chamber (Torr)

$\beta$  = the oxygen capacitance at the selected temperature ( $\mu\text{mol L}^{-1}\text{Torr}^{-1}$ ) (from Dejours 1975)

$V$  = water volume of chamber minus embryo and yolk (L)

$M$  = whole embryonic wet mass (minus external yolk) (g)

$t$  = the interval between sampling (min)

At the termination of the experiment, the lid was removed and the embryo weighed (to the nearest 0.1 g) (Chapter 3, section 3.2.2, p. 66) and replaced into its natural egg capsule. The volume of water in the respirometer was measured to the nearest 5 ml with volumetric cylinders.

### **5.2.1.3 Open respirometry**

#### **5.2.1.3.1 Experimental design**

The experimental design is shown in Figure 5.1. Seawater was pumped with a peristaltic pump (Cole Parmer Masterflex) from the respirometer into a flowmeter (Gilmont H205 or H273) and directed to the oxygen electrode which was positioned in the aquarium itself so that it was equilibrated with the experimental temperature. The electrode was connected to a Radiometer BMS2-Mk3 gas analyser and  $PO_2$  was recorded on a chart recorder. Water was finally channelled back into the aquarium. All tubing was polyvinylchloride with a 3 mm inner diameter. Flow rates varied between 5-20 ml/min such that the difference in  $PO_2$  between inflow and outflow water was approximately 30 Torr. Water could be selectively pumped from the aquarium or from the respirometer by way of a three way stopcock. The electrode was initially calibrated at the start of each day, by the addition of zero solution directly to the electrode. This was flushed out and aerated water pumped through the electrode to set the range.

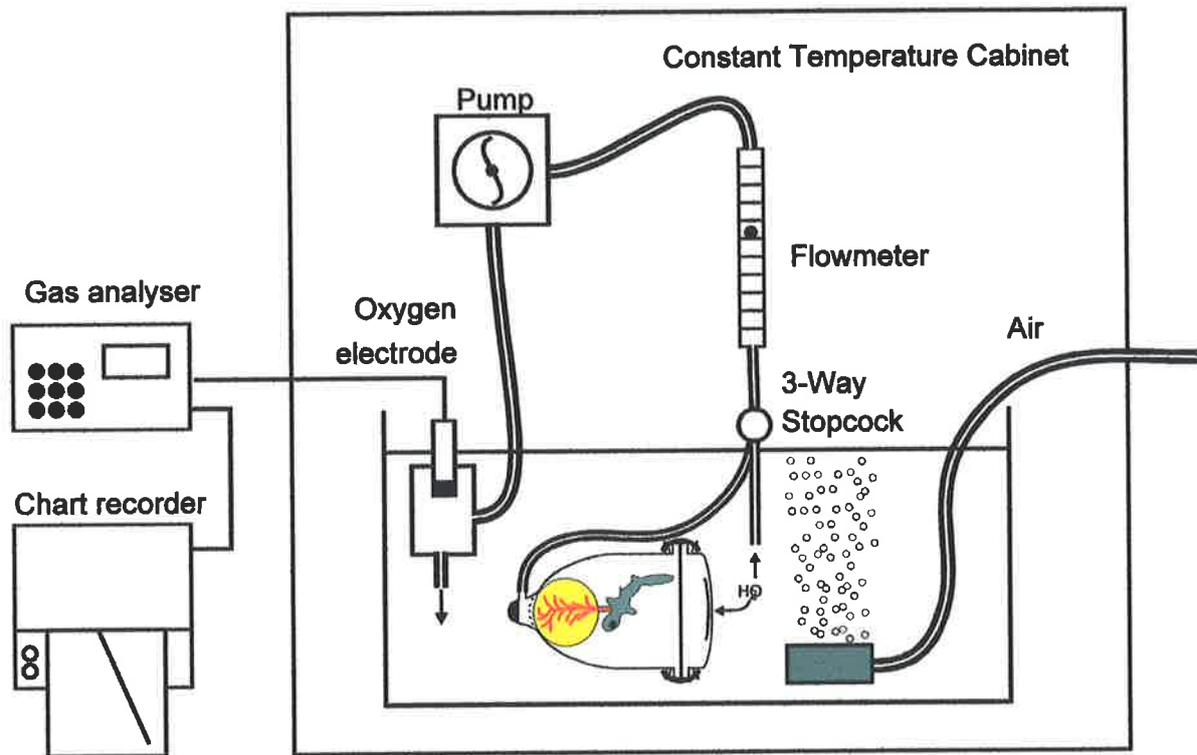


Figure 5.1 Experimental design for open respirometry.

Aquaria were placed in a constant temperature cabinet and the oxygen levels were maintained at saturation with the use of several air stones. All measurements were taken during daylight hours, and the aquarium was covered with black plastic so that disturbance was minimal and the dark conditions inside the egg capsule were mimicked. Once the electrode was calibrated, the respirometer chambers were flushed continuously for 2-3 h before measurements were recorded, in order to establish that the chambers had reached steady state. Recordings of outflow water  $PO_2$  continued for another 2-3 h. At the end of recording, the oxygen electrode was re-calibrated with aerated seawater. Electrode drift during the experiment was assumed to be linear, measured by drawing a line between saturated values at the beginning and end of the experiment. This line represented the incurrent  $PO_2$  value ( $P_iO_2$ ). The excurrent  $PO_2$  ( $P_eO_2$ ) was determined from the chart. Controls were run without embryos for the same period of time to measure background consumption of seawater organisms. The rate of oxygen consumption was determined from the equation:

$$\dot{V}_{O_2} = [(P_e O_2 - P_i O_2) \times \beta \times V_i] / M \quad (\text{eq. 5.3})$$

where  $\dot{V}_{O_2}$  = oxygen consumption of the embryo and seawater ( $\mu\text{mol min}^{-1}\text{g}^{-1}$ )

$P_e O_2$  =  $P_{O_2}$  in the excurrent water (Torr)

$P_i O_2$  = in the incurrent water (Torr)

$\beta$  = the oxygen capacitance at the selected temperature ( $\mu\text{mol L}^{-1}\text{Torr}^{-1}$ )

$V_i$  = flow rate ( $\text{L min}^{-1}$ )

$M$  = whole embryonic wet mass (minus external yolk) (g)

Background consumption was subtracted from the total consumption rates to calculate embryonic metabolism.

#### 5.2.1.3.2 Respiratory chambers

Initial attempts were made to convert natural egg capsules into flow-through respirometer chambers. However, because of the difficulties in seeing the embryo, attaching tubes and electrodes to the capsule and metabolic activity of the capsules external bio-layer, I designed specific respirometer chambers made of glass. The shape of the egg capsule has important consequences on the ventilatory patterns of the embryo, therefore the respirometer was made to mimic the natural egg capsules dimensions as closest as possible. The dimensions of respirometers were estimated by creating a plaster of Paris cast of the inside of an egg capsule. The plaster was prepared and poured into a natural egg capsule and left to cure. The capsule was peeled away, leaving a replicate of the internal shape of an egg capsule, providing dimensions for the glass chamber. In addition, the glass replicate included openings representing the respiratory slits (Figure 5.2). The narrow end contained a slit 1 mm wide, 10-15 mm long and the broad end had a longer slit (40-50 mm long). In all, three respirometers were made. They were 120 mm long and 60 mm at the widest point and the internal volume (minus the embryo) was approximately 150-200 ml. The respirometer was made in two pieces so that the animal could be placed inside with ease. Each segment had two glass hooks equally spaced and directed towards the respective ends. A ground glass interface and elastic bands which were hooked into place from end to end ensured a sealed environment.

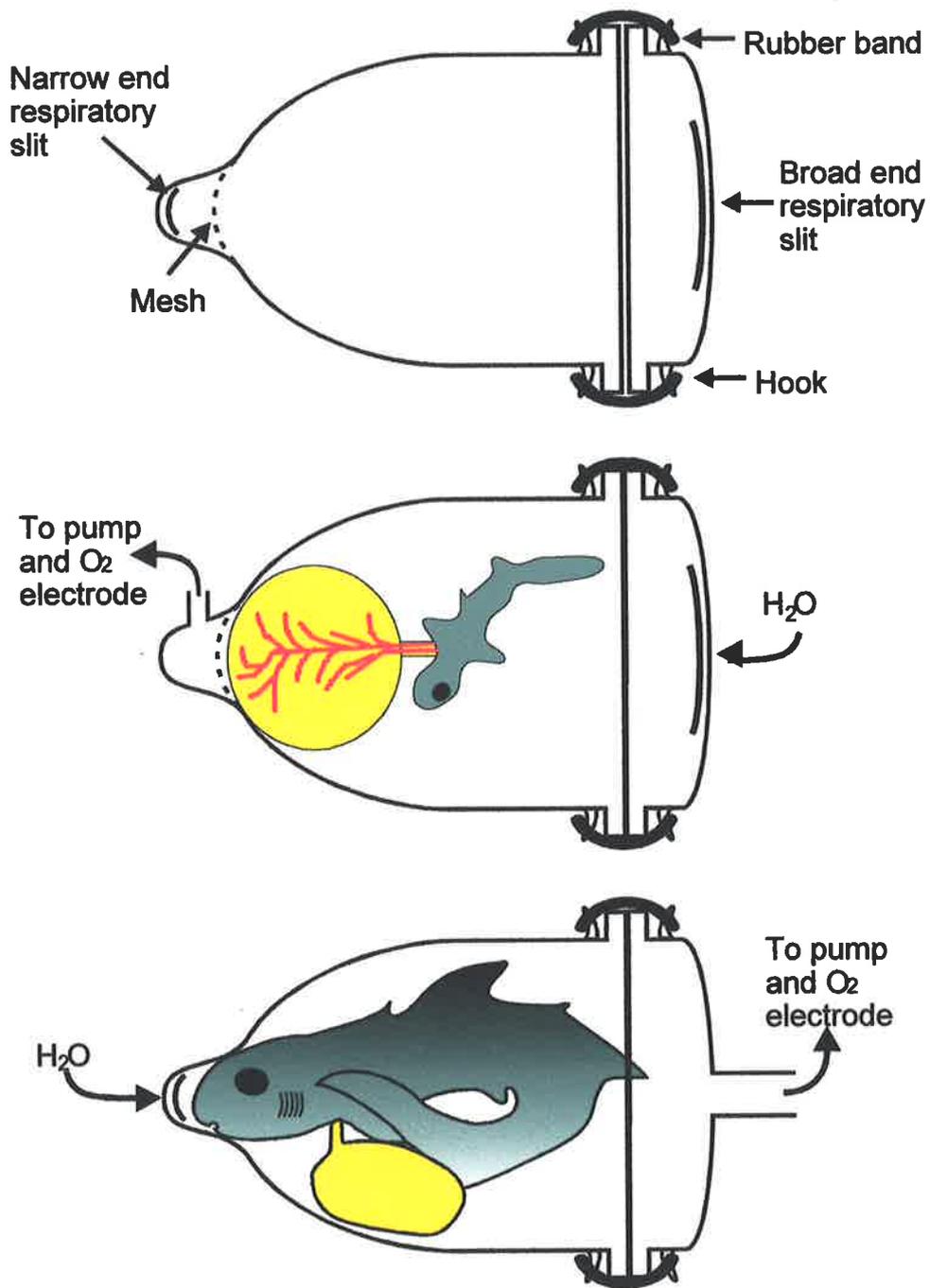


Figure 5.2 Respirometer design for embryos at different stages of development. The top design is the generalised chamber showing the location of the respiratory slits. The middle chamber was designed for small to intermediate embryos. Water is drawn into the chamber through the broad end and out through the narrow end by a peristaltic pump. In larger embryos, the bottom design was used in which water was drawn in through the narrow end and out through the broad end.

Two respirometer designs were used, depending on the stage of development of the embryo. Embryos younger than stage 13 ventilated their eggs by drawing water in through the broad opening and out through either the narrow or broad opening. Older embryos (stage 13 onwards) placed their snout in the narrow end of the capsule and drew water in through the narrow end and out through the broad end. This had consequences for the design of in- and outflow tubes for the different stages. In the situation for young embryos, inflow water was pumped in through the broad end and drawn out at the narrow end (Figure 5.2). A fine mesh was placed at the narrow end to prevent the yolk sac from blocking the exhalant flow. The slit was covered with a rubber stopper so that the exhalant water flow was from one controlled exit point. Older embryos were provided with an inflow at the narrow end, and water was pumped out from a modified broad end (Figure 5.2).

#### **5.2.1.3.3 Embryonic preparation**

The upper section (broad side) of an egg capsule was cut open with a razor blade and a large slit made around two-thirds of the circumference of the case. This facilitated the removal of the animal and replacement back into its own egg capsule at the termination of the experiment. Embryos were removed from their capsule and weighed (minus external yolk - see Fig. 3.1, Chapter 3, section 3.2.2, p. 66). The embryo was placed into the respirometer underwater, and the two segments were joined. At least 16 h prior to experimentation was allowed for acclimatisation, during which period the embryo was able to ventilate the respirometer itself through the open respiratory slits.

#### **5.2.2 Critical $PO_2$**

Some closed respirometry experiments were continued below a  $PO_2$  of 80 Torr, in order to determine the critical  $PO_2$  ( $P_c$ ) for the embryo. The  $P_c$  was the  $PO_2$  at which the embryo no longer maintained a oxygen consumption independent of ambient  $PO_2$ . The  $PO_2$  fell to approximately 20 Torr before the experiment was terminated.

Data from nine stage 12 individuals were analysed to determine the  $P_c$ . A dual linear regression analysis was performed in which two linear regressions are fitted to the data by calculating the least combined residual sum of squares value for two sets of data points (Yeager and Ultsch 1989).

### **5.2.3 PO<sub>2</sub> inside the natural egg capsule**

Six stage 11-12 embryos were used to determine the natural lower PO<sub>2</sub> threshold inside a capsule before the embryo flushes it with fresh seawater. Each egg was placed in an aquarium in a constant temperature cabinet. The seawater was aerated with several air stones. The embryo was free to flush the capsule when ever it chose through the naturally occurring respiratory slits. Two tubes of polyvinylchloride (3 mm inner diameter) were inserted 2 mm into the capsule near the broad and narrow respiratory slits. Seawater was pumped from the capsule with a peristaltic pump (20ml/min) through a loop that consisted of a flowmeter, an oxygen electrode and back into the capsule. The oxygen electrode was connected to a Radiometer BMS2-Mk3 gas analyser and the PO<sub>2</sub> inside the capsule was recorded on a chart recorder. The experiment was repeated five times at 18, 20 and 22°C.

### **5.2.4 Gas exchange in the sealed egg capsule**

Oxygen consumption measurements were not possible while the capsule was sealed because the embryo died if the capsule was prematurely opened. Therefore I examined the morphological features of stage 4-10 embryos (Chapter 2, section 2.3.6, p. 25) and measured the PO<sub>2</sub> of the albumen to infer the status of gas exchange in the first 4 months of incubation. In addition, the respiratory role of the external gill filaments was assessed.

#### **5.2.4.1 Albumen PO<sub>2</sub>**

Eleven eggs (between stage 1-10) were opened prematurely and analysed for albumen oxygen levels. The egg capsule was cut such that the top third was completely removed. Samples of albumen (thick and thin) were removed with a 5 ml syringe immediately on opening. Air bubbles were excluded from the syringe by removing the albumen sample slowly. The syringe was sealed immediately after sampling, the sample location recorded and the PO<sub>2</sub> of the sample analysed within 1-2 min of removal. The thick albumen was located on the outside of the thin albumen and closest to the egg capsule. Depending on the amount of thick and thin albumen that was available, between 1-3 samples were taken from each egg and averaged. Samples of thin albumen were taken above the embryo, close to the broadside slit.

#### 5.2.4.2 Role of external gills

I attempted to establish if the embryo was able to regulate the blood flow through the external gill filaments. Since this could not be done on embryos in sealed capsules (because of premature death), I used stage 11 embryos from capsules just recently opened. The regulatory function of the external gill filaments was assessed by examining the blood flow through the filaments under normoxic, hypoxic and anoxic conditions. Three stage 11 embryos (1-2 g wet mass) with extensive gill filaments were removed from their recently opened capsules and examined in a special holder (Figure 5.3). They were placed individually in large, deep glass petri dishes (10 cm diameter) and covered with aerated seawater. For the examination of blood flow, the embryo had to be kept as still as possible and was therefore lightly anaesthetised by adding 5 mg MS-222 per g body mass to one litre of seawater surrounding the embryo. This level of anaesthetic was sufficient to reduce body movement without significantly effecting heart rate in larval rainbow trout, *O. mykiss* (Mirkovic and Rombough 1998).

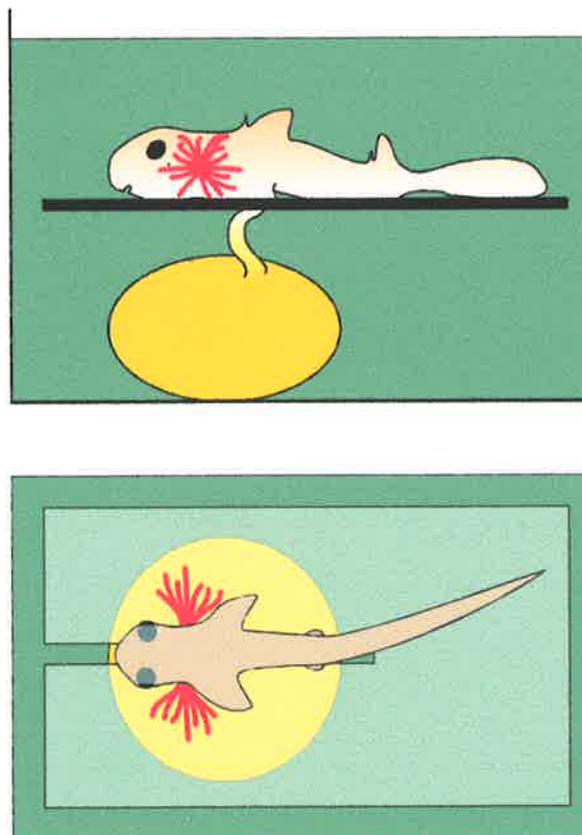


Figure 5.3 Experimental set-up for examination of external gill filaments. The upper diagram represents the side view and the lower diagram the view from the top showing the external filaments spread out.

The embryo was then lifted and placed on a flat plastic acrylic sheet and the gill filaments spread such that they lay relatively flat. The yolk stalk was fitted through a slot (4 mm wide) cut into the acrylic sheet such that the embryo lay above and the yolk sac lay below the sheet. The embryo and yolk stalk were fully submerged in the seawater throughout the experiment. The petri dish was placed under a binocular microscope which was connected to a video camera and recorder. The image was immediately available on a television monitor. The heart rate and blood flow through the filaments were first observed under aerated seawater conditions to determine baseline values. The heart rate was measured by the rate of pulsation of blood through the external filaments. When a stable heart rate was reached, the water was made hypoxic with the addition of 1 ml of a Na<sub>2</sub>SO<sub>3</sub> solution. Anoxia was eventually induced over a period of 5-10 min during which time the blood flow and heart rate were continuously monitored visually. Oxygen tensions were constantly monitored with a Radiometer (Model E5046) oxygen electrode placed in the seawater near the embryo; thus correlation between oxygen levels and heart rate was possible. Eventually anoxic conditions prevailed for 20 min. On termination of the experiment, the embryo was removed from the petri dish and provided with hyperoxic water for 5-10 min, after which it completely revived. The experiment was repeated 2-3 times on the same individuals over a period of one week.

### **5.2.5 Ventilation**

Research on fish ventilation has concentrated on factors affecting both the rate and volume of water movement over the gills in adult and juvenile teleosts, elasmobranchs and hagfish. Factors such as ambient PO<sub>2</sub> (Kerstens et al. 1979, Campagna and Cech 1981), PCO<sub>2</sub> and acid-base parameters (Heisler et al. 1988, Graham et al. 1990) and temperature (Burton 1979, Steffensen et al. 1984) have been investigated. The methodology used to assess these factors is highly variable, including direct writing levers (Lutz 1930), forced displacement and pressure transducers (Satchell 1959, 1960), and electrical impedance changes between two electrodes placed either side of the pharyngeal cavity (Amend et al. 1970, Ferry-Graham 1999). Even the movement of parasitic larvae have been used to observe water flow through different gills in brown trout, *Salmo trutta* (Paling 1968), and the movement of dye to determine the direction of water flow in swell sharks (Ferry-Graham 1999). Non-invasive recording of bioelectrical signals through water has also been used to measure heart rate and

ventilation rate in rainbow trout, *O. mykiss*, eliminating the need for surgery or restraints (Altimiras and Larsen 2000).

Ventilatory movements in the elasmobranch, *H. portusjacksoni*, have been investigated using a piezoelectric flexure element placed in a collar around the buccal area (Capra 1976). Dye-dilution techniques have been employed to measure ventilation in the elasmobranch, *Squalus acanthias* (Millen et al. 1966) and three species of tuna (Bushnell et al. 1990). Alternatively, the use of electromagnetic probes or the collection of water leaving the gills can measure both the rate and volume of water directly entering or leaving the gill chambers (Baumgarten-Schumann and Piiper 1968, Kerstens et al. 1979, Campagna and Cech 1981, Steffensen et al. 1984, Heisler et al. 1988, Graham et al. 1990).

#### **5.2.5.1 Ventilation patterns**

The direction of ventilatory flow through open egg capsules was observed qualitatively using Evans Blue dye. Six eggs representing each of the stages after capsule opening until just prior to hatch (stages 10-14) were placed individually in plastic containers of un-stirred, normoxic seawater held at 20°C in a constant temperature room. The dye was mixed with seawater in a beaker, drawn into a 5 ml syringe and slowly injected near the respiratory slit at either end of the egg capsule. Any movement of dye into or away from the egg capsule was noted. This was repeated twice for each egg.

#### **5.2.5.2 Ventilation rate of capsules**

The ventilation rate through eleven capsules containing stage 12-14 embryos (mass range 13.3-42.5 g) was measured at 20°C using a tracer washout technique. Manganese was chosen as a tracer because it is virtually absent in natural seawater. It was measured with a Varian Atomic Absorption Spectrophotometer (AAS). A standard 1mmol/L (=54.9 g/L) manganese solution was made by adding 0.0845g  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  to 500 ml seawater. Dilutions of the standard were then made to produce 5.49, 1.37, 0.69, 0.137 and 0.069 g/L solutions for instrument calibration (1/10, 1/40, 1/80, 1/400 and 1/800 respectively). The seawater from each egg was gently removed by opening the broad end respiratory slit and pouring the water out. The volume of each egg capsule (minus the embryo and yolk) was determined by measuring the volume of this water (Vint). The eggs were then placed in seawater containing a known manganese concentration which varied between 11-40 g/L, filled with the new manganese seawater solution and

left for 60 min. Eggs were then removed from the high manganese seawater solution and dipped into fresh seawater to wash any manganese solution from the external surface of the egg capsule. They were then placed individually into open plastic containers containing normal aerated seawater of known volume. The broad end of the egg capsule was held closed while the egg was lifted and washed to prevent loss of internal seawater. The external yolk sac often blocked the narrow respiratory slit, preventing the loss of water from the egg. However, if there was a leak of manganese solution from the egg via the respiratory slit, the process of filling and washing was repeated. It was possible that there were traces of the manganese solution on the outside of the capsule when it was placed into the fresh seawater, therefore it was important to measure the initial concentration in the container.

The concentration of manganese in the external water was monitored for 1-2 h. Samples were taken directly from the containers in which the eggs were placed. The water was stirred vigorously before being sampled by the ASS. It was not practical to measure internal manganese concentration during the experiment, because it involved disturbing the embryo for sampling. For the first 30 min, measurements were taken every 1-5 min, followed by less frequent sampling (every 10-30 min) once equilibration between the egg capsule and external water was reached to ensure true equilibrium had been achieved. Ventilation rate ( $\text{ml min}^{-1}\text{kg}^{-1}$ ) was calculated from the following expression :

$$\text{VR} = (\text{Vext} \times ([\text{ext}]_f - [\text{ext}]_i) / ([\text{int}] - [\text{ext}]_i)) / \text{time.mass} \quad (\text{eq. 5.4})$$

where VR = the ventilation rate ( $\text{ml min}^{-1}\text{kg}^{-1}$ )

Vext = the volume of water external to the egg capsule (ml)

[ext]<sub>f</sub> = the equilibrium concentration of Mn in external water (mg/L)

[ext]<sub>i</sub> = the initial concentration of Mn in external water (mg/L)

[int] = the concentration of Mn inside the egg capsule (mg/L)

time = the time between sampling (min)

and mass = wet mass of embryo minus external yolk (kg).

The values for [ext]<sub>i</sub> and [ext]<sub>f</sub> were measured directly from the AAS as concentrations at the beginning and end of a sampling period respectively. The value for [int] was calculated from the following equations:

$$[\text{egg}] = [\text{tot}] - [\text{ext}] \quad (\text{eq. 5.5})$$

$$\text{and } [\text{int}] = [\text{egg}]/V_{\text{int}} \quad (\text{eq. 5.6})$$

where  $[\text{egg}]$  = the total amount of Mn inside the egg capsule (mg)

$[\text{tot}]$  = the total amount of Mn in the entire system (mg)

$[\text{ext}]$  = the total amount of Mn in the external water (mg)

$V_{\text{int}}$  = the internal volume of the egg capsule (L).

The effect of hypoxia on ventilation rate was assessed in six stage 13-14 embryos. The eggs were filled with a manganese solution with low  $\text{PO}_2$  (range = 60-70 Torr) solution of seawater, left for 60 min and placed in fresh hypoxic seawater (range = 60-70 Torr). The water was made hypoxic by bubbling nitrogen gas into seawater. A  $\text{PO}_2$  of 60-70 Torr approximated the critical oxygen pressure at which oxygen consumption became environmentally dependent for stage 12 embryos (section 5.2.2, p. 136). During the experiment, the  $\text{PO}_2$  of the external water was periodically checked with a calibrated Radiometer oxygen electrode (E5046) (section 5.2.1.1, p. 130).

### 5.2.5.3 Ventilation frequency

The frequency of gill ventilatory movements was examined in ten stage 14-15 embryos at three experimental temperatures (18, 20 and 22°C). Ventilation frequency was recorded as changes in impedance across an artificial egg capsule. Two electrodes were constructed by welding a fine insulated wire to a 26 gauge needle and sealing the region of attachment with epoxy glue. The needle point was made blunt with a small amount of glue. The electrodes were placed permanently into either end of a glass respirometer with glue. The wires were connected to a Parks Model 270 impedance plethysmograph which generated a low intensity alternating current field across two electrodes (McDonald et al. 1977). Movement of the embryo changed the impedance of the seawater between the electrodes which was transformed into a voltage output detected on a Grass polygraph 79D. The rate of ventilatory movements was counted against a time scale on the same recorder.

Embryos were removed from their egg capsules, weighed to the nearest 10 mg and placed inside the respirometer. In addition to the impedance electrodes, an oxygen electrode was fitted into the respirometer such that it protruded 10 mm into the internal

volume. The experiment commenced after a period of 12-16 h of equilibration, during which time the respirometers were flushed with aerated seawater at the embryos demand. The  $PO_2$  inside the respirometer was constantly monitored as the embryo depleted the oxygen. This also allowed a measurement of minimal oxygen levels before the embryo flushed out the chamber. Because the respirometers were made of clear glass, the ventilation frequency recorded by the electrodes was verified by a visual count of gill movements. The effect of hypoxia on ventilation frequency was examined.

## 5.3 RESULTS

### 5.3.1 Oxygen consumption of embryos from open capsules

#### 5.3.1.1 Open versus closed respirometry

The logarithmic relationships between mass and the rate of oxygen consumption ( $\text{nmol min}^{-1}$ ) from closed and open respirometer techniques were compared using an ANCOVA statistic. There was no significant difference in the slopes ( $F=1.484$ ) or the intercepts ( $F=0.144$ ) at the 1% level. This allowed data from both methods to be combined in subsequent analysis, with the exception of evaluation of the  $P_c$  which was determined from closed respirometry alone.

#### 5.3.1.2 Effect of embryonic wet mass

Oxygen consumption was measured over a range of embryonic wet masses (minus external yolk) (1.286-40.10 g at 15°C), (9.115- 54.60 g at 18°C), (0.72-59.1g at 20°C) and (7.769-58.7g at 22°C) (Figure 5.4).

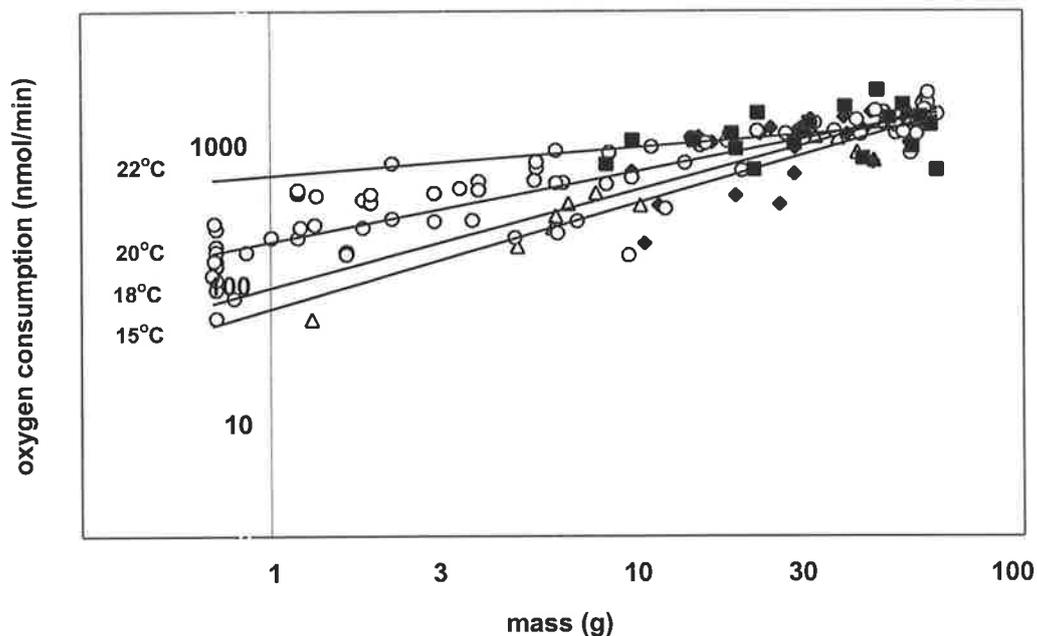


Figure 5.4 The relationship between oxygen consumption and mass on a log-log scale at 15 (open triangles), 18 (solid diamonds), 20 (open circles) and 22°C (solid squares).

The following allometric equations were derived from the double logarithmic transformation of the data. An ANCOVA showed significant differences in the slopes ( $F=1947.0$ ) at the 1% level.

$$15^{\circ}\text{C} \quad \dot{V}\text{O}_2 = 53.58 M^{0.831} \quad r^2=0.909 \quad n=11, T=9.496, P<0.001 \quad (\text{eq. 5.7})$$

$$18^{\circ}\text{C} \quad \dot{V}\text{O}_2 = 77.09 M^{0.769} \quad r^2=0.467 \quad n=25, T=4.486, P<0.001 \quad (\text{eq. 5.8})$$

$$20^{\circ}\text{C} \quad \dot{V}\text{O}_2 = 173.42 M^{0.557} \quad r^2=0.818 \quad n=59, T=16.304, P<0.001 \quad (\text{eq. 5.9})$$

$$22^{\circ}\text{C} \quad \dot{V}\text{O}_2 = 557.19 M^{0.223} \quad r^2=0.117 \quad n=19, T=1.503, P>0.05 \quad (\text{eq. 5.10})$$

where  $\dot{V}\text{O}_2$  = rate of oxygen consumption ( $\text{nmol min}^{-1}$ )

and M = embryonic wet mass (minus external yolk) (g).

### 5.3.1.3 External vs internal gill respiration

Data at  $20^{\circ}\text{C}$  was separated on the basis of oxygen consumption of embryos with extensive external gill filaments ( $< 4$  g wet mass) and embryos using internal gills and having no external gill filaments (Figure 5.5).

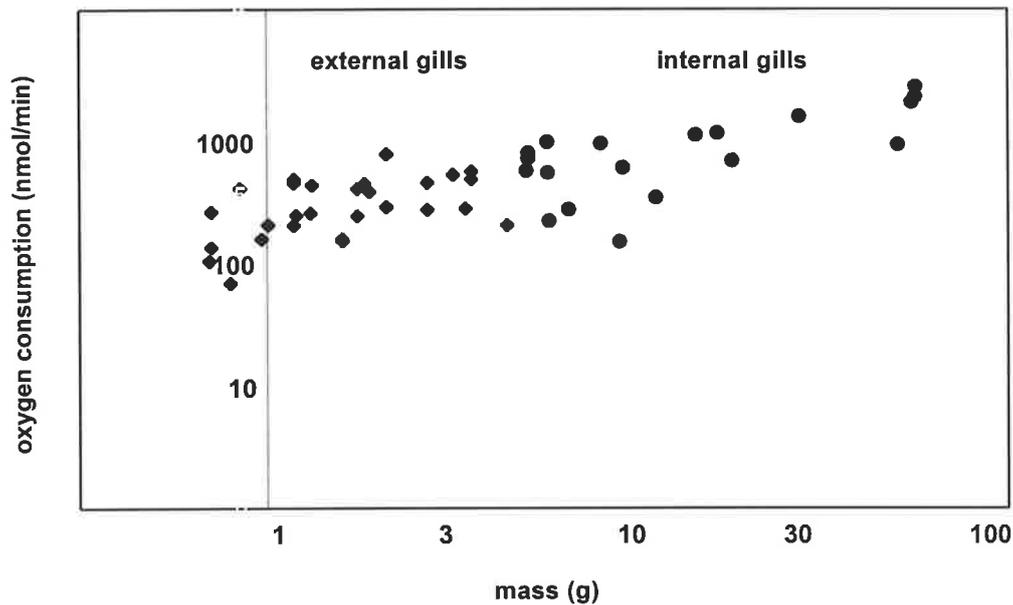


Figure 5.5 The relationship between oxygen consumption and mass in embryos with (solid diamonds) and without external gills (solid circles) on a log-log scale.

Regression analysis for the two data sets were performed and compared with an ANCOVA. There was no significant difference between the two regressions at the 5% level ( $F = 1.655$ ).

$$\text{external gills} \quad \dot{V}O_2 = 162.8 M^{0.879} \quad r^2=0.522 \quad n=36 \quad (\text{eq. 5.11})$$

$$\text{internal gills} \quad \dot{V}O_2 = 134.8 M^{0.621} \quad r^2=0.499 \quad n=21 \quad (\text{eq. 5.12})$$

where  $\dot{V}O_2$  = rate of oxygen consumption (nmol min<sup>-1</sup>) and M = embryonic wet mass (minus external yolk) (g).

#### 5.3.1.4 Effect of temperature

From the equations relating oxygen consumption and mass, it can be shown that as temperatures increase, the value of the mass coefficient "a" increased, representing a significant increase in elevation of slope as temperature rose (F=43.1). Values of "a" were 53.58, 77.09, 173.42 and 557.19 for increasing temperatures.

The oxygen consumption of an embryo of standard mass was estimated at 15°C and 22°C from equations 5.7 and 5.10 (Table 5.1), and the Q<sub>10</sub> of metabolism calculated using equation 5.13 (Dejours 1975) :

$$Q_{10} = \left( \frac{VO_{2t2}}{VO_{2t1}} \right)^{\frac{10}{t_2-t_1}} \quad (\text{eq. 5.13})$$

Temp. (°C)	Standard mass (g)				
	5.0	10.0	20.0	30.0	50.0
15	204	363	645	904	1383
22	797	931	1087	1189	1333
Q <sub>10</sub>	7.00	3.84	2.11	1.48	0.95

Table 5.1 Q<sub>10</sub> of metabolism for standard embryonic masses between 15 and 22°C. Values inside the table are calculated oxygen consumption rates (in nmol min<sup>-1</sup>) from equations 5.7 and 5.10. Q<sub>10</sub> was then calculated using equation 5.13.

#### 5.3.1.5 Critical PO<sub>2</sub>

Figure 5.6 demonstrates the effect of decreasing oxygen tension on the oxygen consumption at 20°C for nine stage 12 embryos in the mass range 9-14 g. The data appeared to be represented by a curve or by two lines. A dual regression analysis produced the following linear equations for the data. Equation 5.14 describes the data at low PO<sub>2</sub>, while equation 5.15 refers to data at higher PO<sub>2</sub>. The point at which the two regressions converge was the PC (= 70 Torr).

$$\dot{V}O_2 = 0.00632 + 0.00102 PO_2 \quad n=12, r^2=0.149, SE_b=0.00077 \quad (\text{eq. 5.14})$$

$$\dot{V}O_2 = 0.05544 + 0.000317 PO_2 \quad n=33, r^2=0.274, SE_b=0.000093 \quad (\text{eq. 5.15})$$

where  $\dot{V}O_2$  = rate of oxygen consumption ( $\mu\text{mol min}^{-1}\text{g}^{-1}$ ) and  $PO_2$  is the ambient partial pressure of oxygen (Torr).

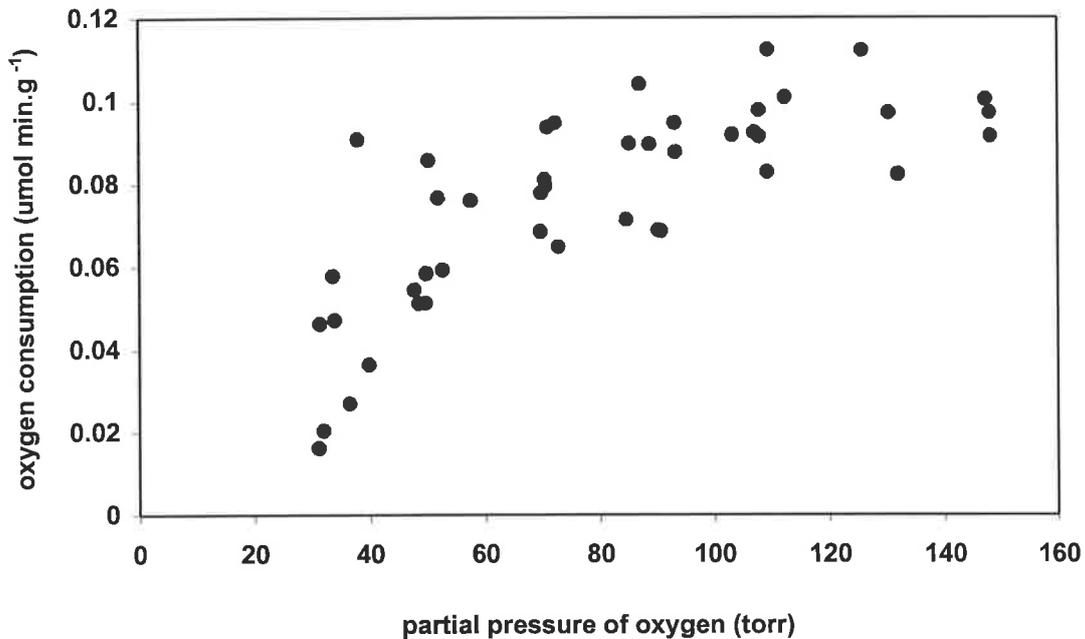


Figure 5.6 Evaluation of the critical  $PO_2$  from closed respirometry results at  $20^\circ\text{C}$ .

However, the calculation of a single value of critical  $PO_2$  is not realistic. The regulation of the oxygen consumption rate does not fail at a precise ambient  $PO_2$ , as indicated by the calculation of two distinct regression lines producing a single  $P_c$  value. The underlying biological components are not integrated into the statistics. The real relationship is a smooth transition from a true regulator to a conformer over a range of  $PO_2$ 's between 60-70 Torr, with the animal making adjustments as the  $PO_2$  decreases. In this case, the regression line above the  $P_c$  (eq. 5.15) was not significantly independent from zero ( $F=11.68, P>0.01$ ), suggesting that oxygen consumption was dependent on  $PO_2$  to some degree. A smooth curve drawn through the data would be a more representative description of the relationship between oxygen consumption and  $PO_2$ .

### 5.3.2 Lower threshold values for $PO_2$ in natural egg capsules

The egg capsule was not ventilated constantly, therefore the  $PO_2$  inside the capsule decreased as the embryo used up oxygen and then quickly increased as the embryo

flushed the capsule with fresh, normoxic water. The average value for the lowest PO<sub>2</sub> and the actual lowest PO<sub>2</sub> recorded in any experiment are presented in Table 5.2. The average for all temperatures fell above the P<sub>c</sub> (70 Torr), although the PO<sub>2</sub> did fall well below the P<sub>c</sub> on some occasions.

temperature	mean ± SE (n)	lowest PO <sub>2</sub> (Torr)
18°C	83.71 ± 7.38 (4)	37.1
20°C	77.28 ± 7.94 (6)	42.6
22°C	81.19 ± 8.98 (5)	58.1

Table 5.2 Lower threshold values for PO<sub>2</sub> inside an egg capsule at 3 temperatures.

### 5.3.3 Gas exchange in the sealed egg capsule : albumen oxygen tensions

The PO<sub>2</sub> of thick albumen in unopened egg capsules was somewhat lower than saturated seawater (150-160 Torr). The PO<sub>2</sub> of thin albumen was markedly lower than both saturated seawater and thick albumen (Table 5.3), and decreased with increasing age.

Embryo length (mm)	embryonic stage	PO <sub>2</sub> thick albumen (Torr)	PO <sub>2</sub> thin albumen (Torr)
0	1	132.5	-
0	1	125	120
7.5	4	149	-
8.3	4	152.5	-
13.8	5	128	-
15.5	5	140	90
29.5	7	-	83
30	7	-	80
36.5	9	-	71.5
37	9	-	70
43.5	10	none	46, 29*

Table 5.3 Mean albumen oxygen pressures at selected stages before egg capsule opening. After stage 7, the thick albumen was absent or difficult to sample because it was located as a thin boundary around the inside lining of the capsule. Where data were not taken is indicated by -, and \* is the mean PO<sub>2</sub> of two samples taken immediately adjacent to the embryo.

### **5.3.3.1 Role of external gills : effect of hypoxia and anoxia on heart rate**

The resting heart rate of normoxic stage 11 embryos was  $80.0 \pm 2.92$  (n=8) beats per min. There was no difference in the heart rate of stage 11 embryos exposed to normoxic, hypoxic and anoxic water even after a 20 min period of exposure to the latter (T=0.464 P>0.05).

## **5.3.4 Ventilation**

### **5.3.4.1 Ventilation patterns**

Patterns of ventilation show considerable variation during the incubation period depending on the stage of development and demand for gas exchange. This is largely related to the relative size of the embryo and the volume of fluid (albumen or seawater) that surrounds it within the capsule. Embryos younger than stage 10 (i.e. those in a closed egg capsule) have a large volume of fluid through which oxygen must diffuse. Thrashing movements of the embryo create conductive currents which increases  $PO_2$  immediately around them. When the capsule opens, the thrashing movements continue in order to exchange seawater between the capsule and the surrounding environment. The end of the caudal fin is modified to form a spoon shape which aids in the movement of water through the capsule. As the embryo grows, it begins to curl around itself, taking up most of the internal volume of the capsule. When there is no room for the embryo to thrash its tail, it places its snout at the narrow end respiratory slit and pumps water directly in through the slit and over the gills.

Direct observations of the movement of Evans Blue dye demonstrated that thrashing movements of stage 10-12 embryos directed water into the egg capsule through the respiratory slit at the broad end of the case, and expelled it through either the narrow or broad openings (Figure 5.7). Older embryos (stage 13-14) were observed to direct water in through the slit at the narrow end of the capsule and out through the broad end.

### **5.3.4.2 Ventilation rate of the capsule**

*H. portusjacksoni* did not ventilate its gills continuously and therefore the flow of water through the egg capsule was variable. The shark exhibited periodic cessations of all ventilatory movements (ventilatory pauses) (c.f. Capra 1976). This created problems when measuring ventilation rate using a tracer washout technique because the dilution was not a smooth function with time. From measurements of the ventilatory frequency I recorded pauses that lasted up to 2 min and occupied up to 8.1% of the overall

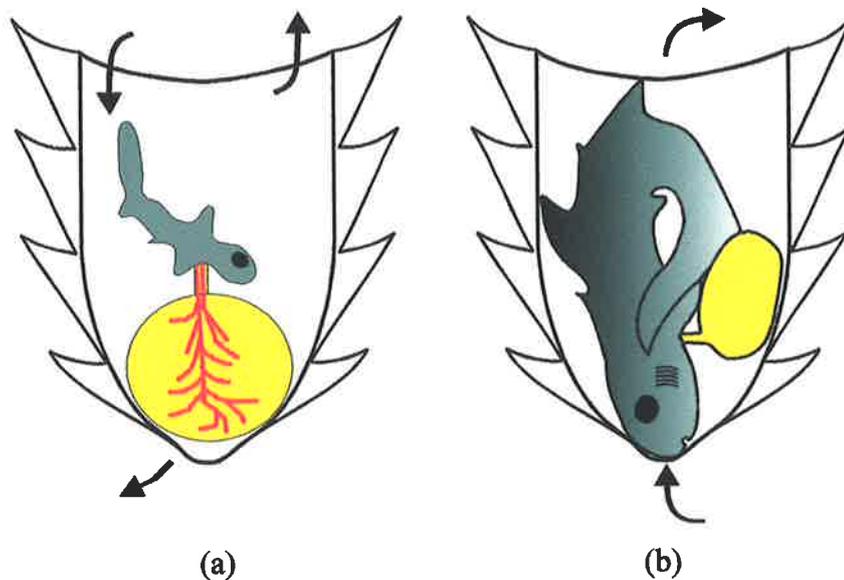


Figure 5.7 Direction of water flow (see arrows) through the capsule in stage 10-12 (a) and stage 13-14 (b) embryos.

ventilatory pattern. In order to calculate an egg ventilation rate, an average rate was calculated from the data including periods where a pause had obviously occurred. Table 5.4 reports the mean ventilation rate during normoxic (150 Torr) and hypoxic (60-70 Torr) exposure in stage 12-14 embryos. More specifically, the ventilation rate of stage 12 embryos (indicated by an asterix) averages  $838.43 \text{ ml min}^{-1} \text{ kg}^{-1}$  or  $12.2 \text{ ml min}^{-1}$ . Stage 13-14 embryos ventilated at  $735.32 \text{ ml min}^{-1} \text{ kg}^{-1}$  during normoxia, and this was increased to  $937.57 \text{ ml min}^{-1} \text{ kg}^{-1}$  during hypoxia. This was equivalent to  $30.01 \text{ ml min}^{-1}$  and  $35.94 \text{ ml min}^{-1}$  respectively. There was no significant difference in ventilation rate at the two oxygen regimes ( $t=0.210$ ,  $P>0.05$ ).

### 5.3.4.3 Ventilation frequency

There was no significant change in frequency with  $\text{PO}_2$  at 18, 20 and  $22^\circ\text{C}$  respectively (ANOVA:  $T=0.246$ ,  $0.456$  and  $0.245$ ,  $P>0.05$ ) (Figure 5.8). However ventilation frequency significantly increased with increasing temperatures (ANCOVA:  $F=53.667$ ,  $P<0.001$ ). The equations below describe the linear regression from these data.

$$18^\circ\text{C} \quad V_f = 74.178 + 0.016 \text{ PO}_2 \quad n=13 \quad r^2=0.006 \quad (\text{eq. 5.16})$$

$$20^\circ\text{C} \quad V_f = 77.739 + 0.017 \text{ PO}_2 \quad n=17 \quad r^2=0.001 \quad (\text{eq. 5.17})$$

$$22^\circ\text{C} \quad V_f = 99.509 - 0.024 \text{ PO}_2 \quad n=16 \quad r^2=0.004 \quad (\text{eq. 5.18})$$

where  $V_f$  = ventilation frequency (beats per min) and  $\text{PO}_2$  = the partial pressure of oxygen (Torr).

Mass-specific ventilation rate	
normoxia	hypoxia
948.2 *	586.7
451.6 *	437.4
601.3 *	1445.0
841.5 *	910.9
1407.6 *	1334.2
780.3 *	911.3
556.5	
854.0	
472.1	
965.9	
828.1	
Overall mean $\pm$ SE (n) = 791.57 $\pm$ 82.65 (11)	mean $\pm$ SE (n) = 937.57 $\pm$ 162.24 (6)

Table 5.4 Ventilation rate ( $\text{ml min}^{-1} \text{kg}^{-1}$ ) of stage 12-14 embryos (mass range 13.3-42.5 g) during normoxia (150 Torr) and hypoxia (60-70 Torr). \* represents data from stage 12 embryos. The remaining data are from stage 13-14 embryos.

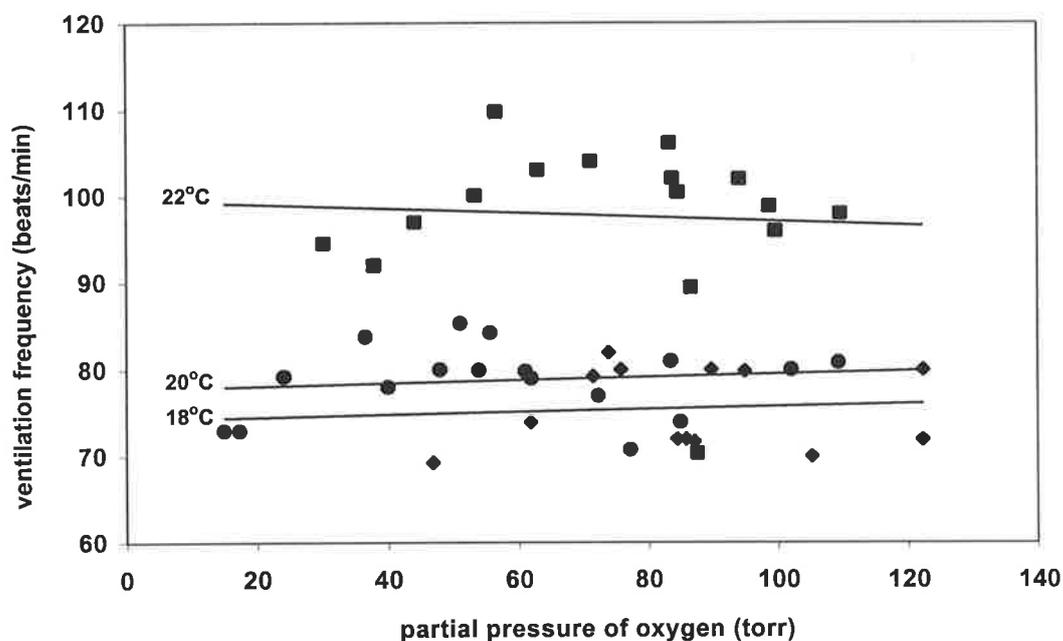


Figure 5.8 The relationship between ventilation frequency (beats per minute) and  $\text{PO}_2$  at 18 (diamonds), 20 (circles) and 22°C (squares).

## 5.4 DISCUSSION

### 5.4.1 Oxygen consumption

The oxygen consumption of hatchling *H. portusjacksoni* sharks ( $0.03 \mu\text{mol O}_2 \text{ g}^{-1}\text{min}^{-1}$  at  $20^\circ\text{C}$ ) falls at the lower end of reported metabolic values for adult elasmobranchs ( $0.025\text{-}0.074 \mu\text{mol O}_2 \text{ g}^{-1}\text{min}^{-1}$ ) (Table 5.5). The mass-specific oxygen consumption decreases as animal mass increases. *H. portusjacksoni* is a sluggish species compared to many of the species represented. This is reflected in the lower than expected mass-specific oxygen consumption for a recently hatched shark compared to adult sharks of different species. Unfortunately intraspecific ontogenic relationships such as this have rarely been explored. A comparison of the oxygen consumptions of embryonic (30-44 g) and adult (390 g) *Heterodontus francisci* was reported by Arnold (1989) in an unpublished Honours project. It was found that at  $18^\circ\text{C}$  embryonic oxygen consumption ( $0.023 \mu\text{mol O}_2 \text{ g}^{-1}\text{min}^{-1}$ , mass=35 g) was markedly higher than adult oxygen consumption ( $0.019 \mu\text{mol O}_2 \text{ g}^{-1}\text{min}^{-1}$ , mass=390 g). Oxygen consumption rates of  $0.076 \mu\text{mol O}_2 \text{ g}^{-1}\text{min}^{-1}$  at  $15^\circ\text{C}$  for hatched dogfish, *S. canicula*, (Diez and Davenport 1987), are twice that reported for adults of the same species at the same temperature ( $0.036 \mu\text{mol O}_2 \text{ g}^{-1}\text{min}^{-1}$  Short et al. 1979). Interspecific comparisons of embryos of similar body mass (14 g) demonstrate that *H. portusjacksoni* consumes oxygen at a similar rate to sandbar sharks, *Carcharhinus plumbeus* and *H. francisci* of  $0.06$ ,  $0.071$  and  $0.068 \mu\text{mol O}_2 \text{ g}^{-1}\text{min}^{-1}$  respectively (Baranes and Wendling 1981, Arnold 1989, this study).

#### 5.4.1.1 Effect of body mass

This study reports mass exponents of  $0.83$ ,  $0.76$ ,  $0.56$  and  $0.22$  at  $15$ ,  $18$ ,  $20$  and  $22^\circ\text{C}$  respectively. The non significant slope at the higher temperature is likely to be due to a combination of the relatively small mass range used ( $7.77\text{-}58.7$  g) and the possibility of increased activity of younger embryos induced by higher temperatures. Metabolic rate in younger stages is more temperature sensitive (Gruber and Weiser 1983, Rombough 1988a, Walsh and Lund 1989, Pepin 1991, Walsh et al. 1991) which may lead to increased activity and oxygen consumption. This will elevate the early phase of the mass-metabolism relationship compared to later stages and decrease the overall slope. Results from the lower temperatures fall within those reported in the literature (Table 5.6).

species	body mass (kg)	metabolic rate (ml O <sub>2</sub> kg <sup>-1</sup> h <sup>-1</sup> )	Temp. (°C)	oxygen consumption at 20°C (μmol O <sub>2</sub> g <sup>-1</sup> min <sup>-1</sup> )	source
<i>Squalus suckleyi</i>	0.1-9	40.2	13	0.051	Pritchard et al. 1958
<i>Squalus suckleyi</i>	2.5-6	42.6	11	0.064	Lenfant & Johansen 1966
<i>Squalus suckleyi</i>	1.6-2.5	26.9	10	0.044	Hanson & Johansen 1970
<i>Scyliorhinus canicula</i>	0.15-0.2	53	13	0.068	Hughes & Umezawa 1968
<i>Scyliorhinus canicula</i>	0.79	48.4	15	0.053	Short et al. 1979
<i>Scyliorhinus stellaris</i>	0.24-3.9	40.2	17	0.037	Piiper & Schumann 1967
<i>Scyliorhinus stellaris</i>	?	38.4	16	0.039	Baumgarten-Schumann & Piiper 1968
<i>Scyliorhinus stellaris</i>	2.0-2.8	46.8	17	0.044	Piiper et al. 1970
<i>Scyliorhinus stellaris</i>	2.0-2.9	65.9	18.5	0.055	Piiper et al. 1977
<i>Scyliorhinus stellaris</i>	1-2.5	33.6	16-19	0.030	Randall et al. 1976
<i>Scyliorhinus stellaris</i>	1.75-3.6	26.9	17	0.025	Heisler et al. 1988
<i>Hemiscyllium plagiosum</i>	?	48	23	0.028	Chang & Wong 1978
<i>Negaprion brevirostris</i>	0.8-1.3	95	22	0.060	Bushnell et al. 1989
<i>Triakis semifasciata</i>	2.2-5.8	68	16	0.074	Scharold et al. 1989
<i>Heterodontus portusjacksoni</i>	0.050		20	0.030	This study

Table 5.5 Oxygen consumption among adult elasmobranchs, at true experimental temperature and units, and corrected to 20°C assuming a Q<sub>10</sub> of 2.2, and μmol O<sub>2</sub> g<sup>-1</sup>min<sup>-1</sup> for comparison with *H. portusjacksoni*.

Species	Mass range	form	b	source
<b>Teleosts</b>				
<i>Salmo gairdneri</i>	5-75 g	A	0.8	Fry 1957
<i>S. gairdneri</i>	0.08-7 g	E-J	0.96	Weiser 1985
<i>S. trutta</i>	80-400 g	A	0.88	Beamish 1964a
<i>Salvelinus fontinalis</i>	30-300 g	A	1.05	Beamish 1964a
<i>Cyprinus carpio</i>	30-500 g	A	0.89	Beamish 1964a
<i>Ophidon elongatus</i>	0.2-20 kg	A	0.78	Pritchard et al. 1958
<i>C. carpio</i>	0.64-1.26 kg	A	0.77	Moffitt & Crawshaw 1983
<i>C. carpio</i>	0.079-1.39 kg	A	0.78	Yamamoto 1991
<i>Sarotherodon mossambicus</i>	10-150 g	A	0.62	Caulton 1978
<i>Pimephales promelas</i>	0.47-2.23 g	J-A	1.05	Wares & Igram 1979
<i>Pleuronectes platessa</i>	4-50 g	A	0.63	Jobling 1982
<i>Gilchristella aestuarius</i>	0.06-1.4 g	J-A	0.77	Talbot & Baird 1984
<i>Barbus aenus</i>	4.1-520 g	A	0.69	Eccles 1985
<i>Pomadasys commersonni</i>	0.05-9.5 kg	A	0.71	Du Preez et al. 1986a
<i>Lithognathus mormyrus</i>	14-201 g	A	0.66	Du Preez et al. 1986b
<i>Lithognathus lithognathus</i>	0.05-2.3 kg	A	0.69	Du Preez et al. 1986b
<i>Theragra chalcogramma</i>	6-300 g	J-A	0.76	Paul 1986
<i>Lichia amia</i>	17-358 g	A	0.72	Du Preez 1987
<i>Pomatoschistus minutus</i>	0.41-2.24 g	A	0.61	Petersen and Petersen 1990
<i>Cyprinodon variegatus</i>	0.2-9.3 g	A	0.68	Nordlie et al. 1991
<i>Cichlasoma managuense</i>	0.006-800 g		0.76	Lezama & Günther 1992
<i>Sillago japonica</i>	0.01-3.7 mg	E	0.78	Oozeki and Hirano 1994
<i>Hippoglossus hippoglossus</i>	100-800 µg	E	1.08	Finn et al. 1995
<i>Paralichthys olivaceus</i>	?	E	0.71	Kurokura et al. 1995
<b>Elasmobranchs</b>				
<i>Squalus suckleyi</i>	0.1-9 kg	A	0.74	Pritchard et al. 1958
<i>Scyliorhinus canicula</i>	0.1-3.3 g	E	0.63	Diez & Davenport 1987
<i>Heterodontus francisci</i>	30-390 g	E-J-A	0.69	Arnold 1989
<i>Heterodontus portusjacksoni</i>	9-55 g	E	0.76	this study

Table 5.6 Mass scaling exponents (“b”) of oxygen consumption for teleost and elasmobranch species, showing the range of masses used to calculate “b”. A=Adult, J=juvenile, E=embryo.

Several phases in the metabolic-mass relationship can be identified which coincide with various stages in the life history of organisms (Weiser 1985, Oikawa et al. 1991, Kurokura et al. 1995). Mass exponents are usually higher in the earlier life stages of a species (Houde and Schekter 1983, Rombough 1988a,b, Oikawa et al. 1991), thus lowering the overall slope of the relationship. The greater mass-specific oxygen consumption during the early stages of ontogeny is related to the intense growth, development of respiratory surface area and increased metabolic demand (Sheel and Singh 1981, Morris and North 1984, Oikawa et al. 1991). Early stages of carp, *C. carpio*, are developing systems such as the brain, heart and digestive organs, which have high oxygen requirements. It is only in the later stages in which the locomotor and respiratory systems, which have lower oxygen requirements, develop (Oikawa et al. 1992). Hence the mass-specific metabolism decreases as the embryo mass increases with proportionately heavier, less oxygen consuming organs. Oikawa et al. (1991) demonstrated that post larval sea bream, *Pagrus major*, have a metabolic exponent of 0.95, whereas juvenile and later stages have a value of 0.82. Table 5.6 suggests a similar trend for trout, *S. gairdneri*. Individuals within the mass range 0.08-7.0 g have a mass exponent of 0.96 (Weiser 1985) whereas larger individuals (5-75 g) have an exponent of 0.8 (Fry 1957). A distinct triphasic metabolic-mass relationship in the larval flounder, *Paralichthys olivaceus*, was reported by Kurokura et al. (1995), although no definitive changes in morphology or oxygen requirements could be directly correlated with the initiation of each phase. It is interesting however that Kurokura et al (1995) demonstrated the lowest exponent was during the first phase, a period of intense metabolic activity for a small increase in mass. Phases two and three follow the ontogenic pattern demonstrated in other studies (that is, the exponent decreases with increasing mass). A similar trend of lower exponents in very early developmental stages was observed in the Japanese whiting, *Sillago japonica* (Oozeki and Hirano 1994). They found that the metabolic-mass relationship in embryonic fish was diphasic, with the second phase demonstrating the decreasing mass exponent with increasing mass. In contrast, eggs and larvae of the grubby, *Myoxocephalus aeneus*, and the milkfish, *Chanos chanos*, showed no stage-specific change in metabolic rate throughout development (Walsh et al. 1989, 1991).

Comparative studies on the ontogenic relationship between metabolism and mass in embryonic elasmobranchs are rare (Diez and Davenport 1987, Arnold 1989, A. Gannon

1992). There are far more difficulties in measuring the early metabolism in elasmobranchs due to the problems in raising them outside the egg capsule or maternal parent. Therefore most studies are from embryos already well developed, i.e., after stage 10 in this study, and the equivalent of stage 13 in Arnold (1989). A. Gannon (1992) studied skates that were 24 days old, already having passed through 15 % of incubation. The entire developmental period of *S. canicula* was examined by Diez and Davenport (1987) and I analysed their data in order to determine if the metabolic-mass relationship was linear or comprised of several phases. The data suggested a diphasic relationship, with the first phase exhibiting a higher slope than the second phase (Figure 5.9). The inflection point between the two phases appeared to occur at approximately egg capsule opening.

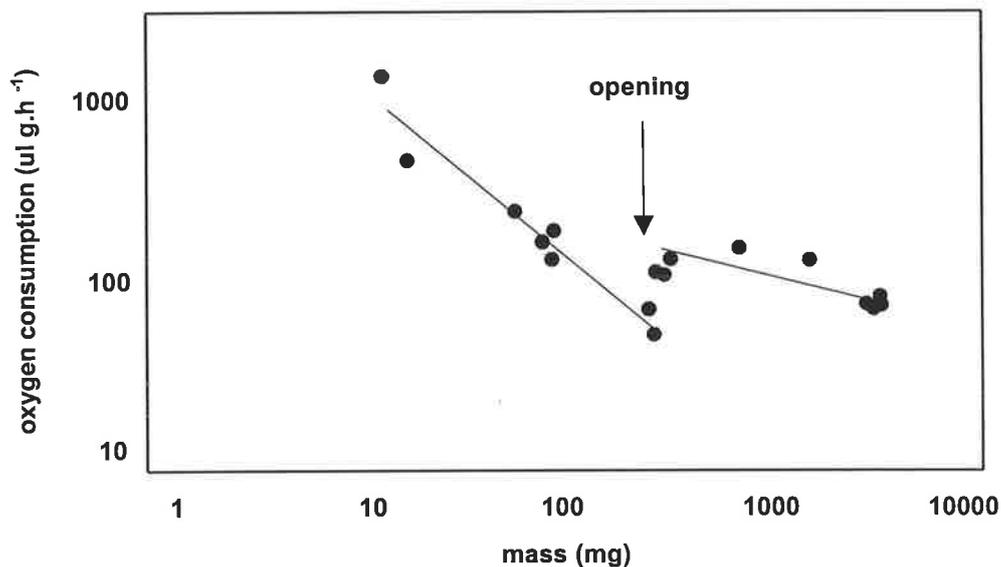


Figure 5.9 Metabolic rate of *S. canicula* showing a diphasic mass-metabolic rate relationship through development.

I was able to examine my data in a similar way although all data were from egg capsules already open. I compared the oxygen consumption from individuals with external gill filaments (stage 11) and without external gill filaments (stage 12 onwards) to determine if gas exchange with external filaments occurs at a different rate than gas exchange without external filaments ( Fig. 5.5, p. 145). I found no significant difference in the exponents relating mass and oxygen consumption, probably because stage 11 embryos

are in the process of reabsorbing the external filaments and are already starting to use the internal gill structure for gas exchange.

It is clear that the relationship between metabolism and mass is not linear through early development, and the use of adult-derived exponents are unlikely to be applicable to early life stages. A generalised mass exponent for all fish species is unrealistic because the variability of exponent values among fish is great (0.29-1.05) (Table 5.6). In addition, to achieve accurate calculations of mass exponents, a large mass range (at least two orders of magnitude) is required (Dejours 1975, Heusner 1982a, Jobling 1983). This overcomes the inherent individual variability due to nutritional and reproductive status, physical health, fitness and age within a species that may affect the oxygen consumption of an animal. However, as Oikawa et al. (1991,1992) point out, mass exponents are stage dependent during embryonic development when such large mass increases occur. Therefore, if only post embryonic forms are utilised in calculation of a mass exponent (in order to eliminate stage effects), it is unlikely that the required mass range will be achieved, thus reducing the accuracy of calculation. Table 5.6 illustrates the variability in mass exponents reported in the literature. The range of masses used in the determination of the mass exponent are given. Rarely do they exceed the desired two orders of magnitude, sometimes they are less than a one order of magnitude difference. This has been the problem with elasmobranch studies to date where the mass range available is too small to determine mass exponents.

#### **5.4.1.2 Effect of temperature**

The relationship between oxygen consumption and temperature is non-linear and may be described by  $Q_{10}$  values (Dejours 1975). Data on adult teleost fish are extensive with values consistently between 1.1 and 3.2 (Table 5.7). Embryonic stages have slightly higher  $Q_{10}$  values, but the temperature ranges used are much smaller, often only 5-6 degree difference.

While the effect of temperature on the oxygen consumption of teleosts has been widely investigated, the  $Q_{10}$  for metabolism in elasmobranchs has been largely ignored. The only elasmobranch study that I could locate calculated a  $Q_{10}$  of 2.1. (7-17°C) in adult dogfish, *S. canicula* (Butler and Taylor 1975). In general, measurements have been carried out at only one experimental temperature, therefore preventing the measurement of  $Q_{10}$ . Moreover the difference in experimental temperatures between studies on a

species is small ( $\approx 3^{\circ}\text{C}$ ) (Table 5.7). The present study reports a  $Q_{10}$  of 0.95 in hatchling sharks, suggesting little effect on oxygen consumption (Table 5.1, p 146) over a  $7^{\circ}\text{C}$  difference.

	$Q_{10}$	$T^{\circ}\text{C}$	Source
<b>Adults</b>			
<i>Salvelinus fontinalis</i>	1.9	10-20	Beamish 1964b
<i>Catostomus commersonii</i>	3.2	10-20	Beamish 1964b
<i>Cyprinus carpio</i>	2.5	10-35	Beamish 1964b
<i>Cyprinus carpio</i>	2.0	10-20	Hughes et al. 1983
<i>Cyprinus carpio</i>	2.6	13-25	Moffitt and Crawshaw 1983
<i>Platichthys stellatus</i>	3.0	11-20	Watters and Smith 1973
<i>Brevoortia tyrannus</i>	2.1	10-25	Hettler 1976
<i>Tilapia rendalli</i>	2.3	17-40	Caulton 1977
<i>Sarotherodon mossambicus</i>	2.3	16-37	Caulton 1978
<i>Mugil cephalus</i>	1.8	13-33	Marais 1978
<i>Liza dumerili</i>	2.2	13-33	Marais 1978
<i>Liza richardsoni</i>	2.0	13-33	Marais 1978
<i>Pleuronectes platessa</i>	1.3	10-20	Jobling 1982
<i>Barbus aeneus</i>	1.1	9.5-26	Eccles 1985
<i>Gilchristella aestuarius</i>	2.3	15-25	Talbot and Baird 1985
<i>Pomadasys commersonni</i>	3.1	15-25	Du Preez et al. 1986a
<i>Lithognathus mormyrus</i>	2.8	10-25	Du Preez et al. 1986b
<i>Lithognathus lithognathus</i>	2.3	10-25	Du Preez et al. 1986b
<i>Theragra chalcogramma</i>	2.5	1-12	Paul 1986
8 sp. mid-water fish	3.2	7-20	Donnelly & Torres 1988
<i>Oreochromis niloticus</i>	1.7	20-35	Fernandes & Rantin 1989
<i>Cichlasoma managuense</i>	2.3	22-32	Lezama & Günther 1992
<i>Ptychocheilus oregonensis</i>	2.4	9-21	Cech et al. 1994
<b>Embryos</b>			
<i>Salvelinus alpinus</i>	4.9	4-8	Gruber & Weiser 1983
<i>Salmo gairdneri</i>	3.0	6-15	Rombough 1988a
<i>Scomber scombrus</i>	1.7	13-19	Giguère et al. 1988
<i>Myoxocephalus octodecemspinosus</i>	2.6	2-8	Walsh & Lund 1989
<i>Mugil cephalus</i>	2.8	24-29	Walsh et al. 1989
<i>Chanos chanos</i>	2.4	28-33	Walsh et al. 1991
<i>Clarias gariepinus</i>	5.6	25-28	Kamler et al. 1994

Table 5.7 Metabolic  $Q_{10}$  values for adult and embryonic teleost fish species.

Temperature usually has the effect on the mass-oxygen consumption relationship of adjusting the elevation of the curve but not the exponent (Hettler 1976, Caulton 1978, Jobling 1982, Degani and Lee-Gallagher 1985, Du Preez et al. 1986, Du Preez 1987). An increase in elevation reflects an increase in oxygen consumption. The results from this study support increased elevation as reflected in higher log transformed Y-intercepts as temperature increases (1.729, 1.889, 2.239 and 2.746). However a decrease in the exponent was also noted at higher temperatures. The consequence of higher intercepts and lower exponents at higher temperatures leads to greater  $Q_{10}$  values for younger embryos compared to older embryos. Table 5.1 (p. 146) demonstrates the decreasing sensitivity of developing *H. portusjacksoni* embryos to the effects of temperature. Embryos with a standard mass of 5.0 g have a  $Q_{10}$  of 7.0, while 50 g embryos had a  $Q_{10}$  of 0.95. It may be that younger embryos at higher temperatures are more sensitive to temperature, and are limited by convection and oxygen supplies. Acute changes in temperature evoke the greatest metabolic response in younger developmental stages of teleosts, suggesting greater sensitivity in these stages compared to older embryos or larvae (Walsh et al. 1991). A change in temperature affected the oxygen consumption of earlier life stages of *C. chanos*, with 13-20 h old stages having a metabolic  $Q_{10}$  of 3.58, while 20-27 h old stages had a  $Q_{10}$  of 1.69. Elevated  $Q_{10}$  values in larval amphibians compared to adults may be the result of a less efficient embryonic system, in particular, with respect to the lower  $Ca^{2+}$  pump efficiency of the heart (Pelster 1999).

#### 5.4.1.3 Effect of hypoxia

As hypoxia is gradually induced, a fish may respond by maintaining its oxygen consumption independent of ambient conditions (metabolic regulator) or it may decrease its oxygen consumption in a linear fashion as oxygen availability falls (metabolic conformers). Few fish can regulate over continuously falling oxygen levels (Mangum and Van Winkle 1973) : there comes a  $PO_2$  at which the fish can no longer maintain its independence and the oxygen consumption rate falls (critical  $PO_2$  or  $P_c$ ). Fish transfer oxygen from the external medium via convection (ventilation) and diffusion across the gill epithelia. The factors controlling the rate of diffusion are the diffusion distance, the surface area, the permeability constant ( $K_{O_2}$ ), and pressure gradients across the respiratory surface. Hypoxia decreases the gradient of oxygen across the gills and therefore the amount of oxygen removed from the water. Metabolic regulators are unperturbed by the decrease in oxygen availability and can maintain their

oxygen consumption at normal levels by making physiological alterations to maintain a constant supply of oxygen to the cells for energy production. This can be achieved in several ways. A greater amount of oxygen can be removed from the medium by increasing the ventilatory flow of water over the gills. In conjunction with increased ventilation, an increase in the cardiac output such that blood flow to the gills is increased and low  $PO_2$  inside the epithelium is maintained. Previously unused secondary lamellae can be opened up to increase the surface area for exchange. This can be achieved by increasing the blood pressure through the gills (Randall 1982a). At the blood level, the affinity of haemoglobin to oxygen can be increased by a reduction of red cell organic phosphate concentration (Wood and Johansen 1972) or the concentration of haemoglobin may increase (Petersen and Petersen 1990).

These physiological alterations, alone or in association with each other, can regulate the amount of oxygen removed from the water. However, this physiological regulation of oxygen uptake has limits. It costs energy to increase the heart work load or the ventilation volumes and this must be offset by the increase in oxygen uptake and hence energy production.

The limiting supply of oxygen is a potentially greater problem in oviparous fish embryos because the eggs are fixed in position and cannot actively move from hypoxic conditions as juvenile and adult fish can. Growth and development are highly metabolically active processes, and to satisfy the embryo's needs, high external oxygen levels are required. During the early stages of development, the energy requirements may be greater than the available yolk vascularisation can supply, so even at air saturation, there may be limited oxygen consumption and therefore retarded growth (Fry 1957). It is also possible that later stages may be more affected by the limitations of the capsule surrounding them (Eldridge et al. 1977, Davenport and Lönning 1980, Davenport 1983, Holliday et al. 1984, Rombough 1988a).

The ability to tolerate conditions of low oxygen may be stage dependent. Early embryos have greater tolerance to hypoxia, even surviving brief exposure to anoxia, compared to older individuals. Early eggs of the lumpfish, *Cyclopterus lumpus*, (up to 6 days old) were able to extract as much oxygen from 10% saturated water as they were from 100% saturated water, whereas 36 day old embryos acted as metabolic conformers and oxygen uptake decreased almost linearly with falling ambient oxygen levels

(Davenport 1983). Rombough (1988a) found that the  $P_c$  for steelhead embryos and alevins increased nine fold as development proceeded. Short exposure at the earliest part of development (prior to gastrulation) in the nase, *Chondrostoma nasus*, produced viable larvae after a prolonged incubation, while exposure later in development resulted in a large percentage of fish dying or hatching deformed (Keckeis et al. 1996). Early dogfish embryos (*S. canicula*) are able to survive for as long as 12 h in anoxic water, becoming anaerobic, while recently hatched individuals were unable to tolerate equal conditions for 30 min (Diez and Davenport 1987). Greater tolerance to hypoxic conditions in early embryos is likely to be due to the lower rates of oxygen demand and to greater oxygen affinity of the blood pigment (Manwell 1963, Iuchi 1973, King 1994, Hamlett et al. 1996). Early embryos also rely on cutaneous respiration for a majority of their respiratory exchange and have greater surface area:volume ratios than older embryos (Rombough 1988b). Therefore oxygen diffusion across the skin becomes more limiting as development proceeds.

Stage 12 *H. portusjacksoni* embryos (between 9-14 g) in open capsules are able to regulate their metabolism to some degree at ambient levels above 70 Torr. Metabolism fell slowly from normoxia (150 Torr) to approximately 70 Torr. Below this, metabolism dropped rapidly in response to progressive environmental hypoxia. Although consumption rates were not measured in earlier embryos (from unopened capsules) data of oxygen tensions within the egg, show that the stage 10 embryo experiences oxygen pressures lower than 50 Torr while the egg capsule is sealed (Table 5.3, p. 148) lending support to the idea that the earlier embryos are more tolerant of hypoxia.

#### **5.4.2 Gas exchange in the closed capsule**

Aquatic embryos take up oxygen from the surrounding water by diffusion through the egg capsule. The maximum size of an egg which relies on gaseous diffusion alone is determined by the diffusion capabilities of the capsule. During development, diffusion is usually sufficient to meet the metabolic requirements because the eggs are generally small, with a corresponding high relative surface area. The degree to which the diffusion may become limiting depends upon the volume of peri-vitelline space and therefore the distance that oxygen must diffuse to get to the developing embryo, and the metabolic requirements of the embryo. Most pelagic teleosts are small and may

encounter diffusion problems near hatching when metabolic demands are high. This is suggested by an increase in metabolism directly after hatch, although this may be a function of increased activity after hatch (Holliday et al. 1964, Eldridge et al. 1977, Davenport and Lönning 1980, Walsh and Lund 1989). The assumption that the capsule limits oxygen diffusion is further refuted by several studies which report no significant rise in oxygen consumption rates after hatch, only the expected increase resulting from greater embryonic mass and enhanced activity (Dabrowski et al 1984, Walsh et al. 1989, Finn 1994). Amphibian eggs differ in that the volume of the capsule changes through development by the selective absorption of water from the environment, thus altering diffusion parameters. In amphibian eggs less than 3 mm in diameter, the ease with which oxygen is transported across the capsule ( $G_{O_2}$ ) increases to match the increasing  $\dot{V}O_2$ , such that the  $\Delta PO_2$  across the capsule during incubation is practically independent of  $\dot{V}O_2$  (Seymour and Bradford 1995). Larger eggs may however experience respiratory difficulties, especially late in development or at higher temperatures. At temperatures above 15°C,  $\dot{V}O_2$  becomes dependent on  $PO_2$  in late stage *Pseudophryne bibronii* and *Crinia georgiana* (Seymour et al. 1991, Seymour and Roberts 1995). Increases in  $\dot{V}O_2$  are observed immediately after hatch in *P. bibronii* (Bradford and Seymour 1985) and other species (Seymour 1994), suggesting a limitation in oxygen diffusion to the embryo or an inability of the embryo to take up enough oxygen while inside the egg.

Oviparous elasmobranch embryos are considerably larger than teleost and amphibian embryos, and they are surrounded by a relatively thick, tertiary egg capsule (Wourms 1977), which may limit the diffusion of respiratory gases to and from the embryo in the early stages of incubation. Two studies (Diez and Davenport 1987, A. Gannon 1992) have shown that oxygen can diffuse through the elasmobranch egg capsule at a rate similar to that of teleost eggs (Rombough 1989). However, as the elasmobranch embryo is much larger (up to several orders of magnitude) diffusion may become limiting as the elasmobranch embryo grows and has an increased oxygen requirement. The capsule of the skate embryo, *R. erinacea*, allowed adequate diffusion for only the younger stages of development (A. Gannon 1992). Once the respiratory slits open, older skate embryos relied on convection to aerate the egg capsule.

The embryo sits atop the highly vascular yolk sac which is not attached to the egg capsule. In the most common oviposition for *H. portusjacksoni* (broadside uppermost),

the yolk sac fits into, and is in direct contact with, the narrow end of the capsule wall. Oxygen diffusing in through this lower part of the capsule has a minimal distance to diffuse across to the yolk circulation, where it is then transported to the very early embryo via the vitelline artery. In any other part of the capsule, the diffusion distance to either yolk sac or embryo is greater because of the presence of albumen. Movement of oxygen within the egg capsule may be facilitated by convection created by embryonic movements, which have been reported to disturb stagnant layers of peri-vitelline jelly surrounding teleost embryos (Walsh et al. 1991). Rhythmic lateral movements occur as early as 15-20% into the incubation of *H. portusjacksoni* (stage 5) and have been recorded in many other teleost and elasmobranch embryos (Clark 1926, Libby 1959, Libby and Gilbert 1960, Rajagopal 1979, Baranes and Wendling 1981, Peterson and Martin-Robichaud 1983, Walsh et al. 1991). Because early vertebrate embryos are partly dependent on cutaneous respiration (Stewart and Castillo 1984, Blaxter 1988), muscular activity is important in reducing the boundary layers which immediately surround the embryo and in disturbing the stagnant albumen (equivalent to the teleost peri-vitelline fluid) which may hinder oxygen diffusion toward the embryo. Movements of the pectoral fins and tail of mountain whitefish, *Prosopium williamsoni*, have been shown to result in extensive mixing of the perivitelline fluid and increased oxygen uptake (Rajagopal 1979). Dye indicators showed that pectoral fin fluttering in embryonic Atlantic salmon, *Salmo salar*, moved the perivitelline fluid from one side of the embryo to the other (Peterson and Martin-Robichaud 1983).

Movement of the teleost embryo within the egg appears to be successful in mixing the capsular fluid enough to improve diffusion, however it is difficult to imagine that such thrashing movements can circulate the entire albumen mass in large oviparous elasmobranch eggs. It may be that diffusion limitations of the egg are the stimulus for the dissolving of the mucous plugs and the opening of the capsule to seawater, thus removing the need for diffusion across the capsule. The inability to increase the oxygen availability to the developing teleost by opening the capsule is the limiting factor on teleost egg size.

The egg capsule has two major roles which become conflicting late in development. It provides protection and support for the embryo but it must allow respiratory gases to diffuse between the environment and embryo. Larger eggs require a more substantial

egg capsule for support but have greater oxygen requirements. Thicker capsules for additional support would only reduce the diffusive capability of the capsule and produce hypoxic environments. Large amphibian eggs would endure the same problems, hence they are limited in size to less than 7 mm diameter (Seymour and Bradford 1995). Oxygen diffusion to amphibian eggs can be aided by the presence of foam (Seymour and Loveridge 1994) or algal symbionts (Pinder and Friet 1994). *H. portusjacksoni* have extremely large eggs (90-208 g) in comparison to teleosts and amphibians. They are able to produce such large eggs because they have overcome diffusion limitations by opening the capsule to seawater approximately 40% into the incubation. The question of whether diffusion can provide enough oxygen to the embryo prior to capsule opening can be examined using known parameters from the Fick diffusion equation and calculating what the difference across the capsule and therefore the intra-capsular PO<sub>2</sub> is under normoxic conditions. I have done this for *S. canicula* using data from Diez and Davenport (1987)(Appendix 2, p. 194). An intra-capsular PO<sub>2</sub> of approximately 40 Torr is comparable to the PO<sub>2</sub> (46 and 29 Torr) measured directly in this study for *H. portusjacksoni* just prior to opening (Table 5.3, p. 148). In both cases this is likely to approach severe hypoxic conditions which may lead to reduced oxygen consumption and growth rates. Diez and Davenport (1987) showed that embryos from open capsules maintained internal PO<sub>2</sub> above 70 Torr, only occasionally falling to 50 Torr over a 6 h period of normoxic exposure. I have similarly demonstrated that the PO<sub>2</sub> inside the open *H. portusjacksoni* capsule generally remains above 70 Torr but can fall to below 40 Torr on occasion (Table 5.2, p. 148, Appendix 4, p. 200) which has negative consequences on  $\dot{V}O_2$  (Figure 5.6, p. 147). Therefore opening the capsule at this time reduces respiratory distress by eliminating the limitations of gas exchange created by the capsule. Similarly, the opening of the capsule of the older skate embryo, *R. erinacea*, removed the problems created by limited oxygen diffusion (A. Gannon 1992).

#### **5.4.2.1 Aerobic or anaerobic?**

There are only a few previous studies of early elasmobranch oxygen consumption (Diez and Davenport 1987, Arnold 1989, A. Gannon 1992, Tullis and Peterson 1997). This may be due to difficulties in keeping embryos alive long enough to make measurements because when the capsule is opened before the mucous plugs have dissolved, the embryo dies within a week (Libby 1959, Read 1968, Ballard et al. 1993, this study), presumably from bacterial infection. In my study, metabolism in embryos younger

than stage 10 was inferred from the morphology of the embryo, direct measurement of oxygen partial pressures inside the capsule and by extrapolating metabolic data from older embryos back to pre-egg capsule opening. Morphological evidence such as the early appearance of circulating blood cells (stage 6) and external gill filaments (stage 7) suggest an aerobic existence in the early *H. portusjacksoni* embryo (chapter 2, section 2.3.6, p. 28-29). In addition, the  $PO_2$  of the thin albumen decreases in a linear fashion as incubation proceeds (Table 5.3, p148), suggesting that a larger  $\Delta PO_2$  is required to match the increasing metabolic demands of the growing embryo. By stage 10, the  $PO_2$  is 46 Torr in the thin albumen and only 29 Torr immediately next to the embryo. This constitutes severe hypoxia to the embryo. However, soon after (stage 11) the egg capsule becomes open to seawater, thus increasing the oxygen availability to the embryo through circulation of seawater.

If the early *H. portusjacksoni* embryo is aerobic, the theoretical oxygen consumption during the period in the closed egg capsule can be estimated by assuming that oxygen consumption increases exponentially as time progressed. From Figure 4.5, (chapter 4, p. 108), I calculated an approximate total consumption of 4.3 mmol  $O_2$  while the capsule is sealed. Alternatively, if the metabolic cost of development at 20°C was 16.44 mmol  $O_2$ /g dry mass (Table 4.5, p. 109), and the dry mass of an embryo at 20°C at the time of egg opening was 0.082g (Table 2.4, p. 23, eq. 4.5, p. 103), then 1.35 mmol  $O_2$  is required during this period (Appendix.3.1, p. 195). The latter figure assumes a constant cost of development throughout incubation, however the cost for development to opening may not be the same for all development.

Nevertheless, given a total consumption of 1.35-4.3 mmol  $O_2$  while the egg is sealed, oxygen must be either provided in sufficient quantities at the time of egg laying or it must diffuse through the case during this period, or a combination of the two. The first scenario assumes that the egg capsule is impermeable to oxygen and the female provides an adequate quantity of oxygen for the embryos respiratory needs at the time of laying. This was examined using results from the measurement of  $PO_2$  within sealed *H. portusjacksoni* capsules (Table 5.3, p. 148) and by calculation of initial egg oxygen content (Appendix 3.2, p. 195). Initially (stages 1-5), the egg  $PO_2$  was 130-146 Torr (80-97% of ambient oxygen levels). By stage 7, the  $PO_2$  dropped to 90 Torr (60% of ambient levels). Just prior to egg opening at stage 10, the  $PO_2$  was 46 Torr - only 30%

of ambient levels. The high  $PO_2$  in the albumen at the beginning of incubation negates the possibility of the female providing an oxygen store at egg laying since the  $PO_2$  in the egg is greater than the  $PO_2$  of her fluids (for example 81 Torr in *Scyliorhinus stellaris* - Piiper and Schumann 1967) and she can not produce oxygen. The gradual decrease in  $PO_2$  (Table 5.3, p. 148) reflects the increasing  $\dot{V}O_2$  of the embryo being matched by the increasing  $\Delta PO_2$ . In addition, the total amount of oxygen initially available in a fresh egg (yolk + albumen) can be estimated by making several assumptions (Appendix 3, p. 195). A theoretical initial oxygen content of 0.035 mmol  $O_2$  was calculated. Clearly, there is not enough oxygen available initially to sustain aerobic metabolism over the 120 day period while the egg capsule is sealed. If the embryo is to metabolise aerobically during this period, oxygen must also diffuse into the egg capsule from the environment.

Under normoxic conditions, metabolism in the dogfish embryo, *S. canicula*, was aerobic with no trace of the anaerobic end product lactate in embryo, yolk or external medium (Diez and Davenport 1987). *S. canicula* embryos were capable of anaerobic metabolism when exposed to anoxia for 8 hrs or more, when significant amounts of lactate were found in both the embryo (1.603 mg/g wet mass) and yolk (0.084 mg/g wet mass). Unfortunately the data for this species must have come from embryos in already opened capsules because the embryos were removed from their capsules and placed individually in the respirometers. This would be difficult to achieve in unopened egg capsules without detriment to the embryo. In addition, recovery from prolonged anoxia in *S. canicula* was determined by observations of resumption of breathing (Diez and Davenport 1987), a variable not visually obvious in very young *H. portusjacksoni* embryos which make use of external gill filaments instead of internal gills until stage 11. There were no muscular gill movements observed in younger stages of development.

Embryos and larvae of several teleost fish species have the capability for both aerobic and anaerobic respiration (Pelster 1999). Arctic char, *Salvelinus alpinus*, can survive extended periods (8h) of anoxic exposure and anaerobic respiration continues to make a significant contribution during the reoxygenation period.

#### **5.4.2.2 Egg capsule permeability**

The permeability of elasmobranch egg capsules (both oviparous and ovoviviparous) to osmotic and ionic substances has been examined by many authors (Needham and

Needham 1930, Price and Daiber 1967, Hornsey 1978, Evans and Oikari 1980, Foulley and Mellinger 1980a, 1981, Evans 1981). Only Diez and Davenport (1987) and A. Gannon (1992) have examined the permeability of such capsules to oxygen. The facility of oxygen movement through a given material is defined by Krogh's diffusion coefficient ( $K_{O_2}$ ) (Dejours 1975). The  $K_{O_2}$  values reported for elasmobranch capsules are  $4.93 \times 10^{-6} \text{ nmol cm}^{-1} \text{ s}^{-1} \text{ Torr}^{-1}$  (Diez and Davenport 1987) and  $1.78 \times 10^{-6} \text{ nmol cm}^{-1} \text{ s}^{-1} \text{ Torr}^{-1}$  (A. Gannon 1992). In addition, values for  $K_{O_2}$  in six salmon species ranging from  $1.16\text{-}3.69 \times 10^{-6} \text{ nmol cm}^{-1} \text{ s}^{-1} \text{ Torr}^{-1}$  were estimated from the relationships between critical  $PO_2$  and metabolic rate (Rombough 1989). In the latter study, there were considerable differences in the total pore area in the capsules of each teleost species that were not reflected in the overall permeability, leading to the hypothesis that it is not the teleost egg capsule that controls the permeability to oxygen. The major barrier to oxygen resides in the vitelline membrane and the peri-vitelline fluid. In contrast to these structures, the teleost egg capsule is relatively freely permeable to oxygen.

Diez and Davenport (1987) measured the oxygen tension within the capsule at various stages of development in *S. canicula*. Early stage eggs (30 days old) demonstrate a very slow decline in  $PO_2$  within the capsule over a 6 hour period, reaching 25-35 Torr below that of saturation. At this time, the embryo is less than 0.01 wet mg and consuming a negligible amount of oxygen. However, their results show that while the capsule is sealed, an oxygen consumption of  $7.43 \times 10^{-6} \text{ mmol min}^{-1}$  for the whole egg (yolk + embryo + egg capsule) is able to be maintained by keeping an internal  $PO_2$  of 25-35 Torr below saturation by the diffusion of oxygen from the environment.

I have indirectly examined the permeability of *H. portusjacksoni* egg capsules using albumen  $PO_2$  measurements and metabolic equations. For each stage for which there were albumen  $PO_2$  data (Table 5.3, p.148), I estimated the oxygen consumption rate from the relationship between wet mass and oxygen consumption (eq. 5.9, p.145). I estimated a  $K_{O_2}$  value of  $9.69 \times 10^{-6} \text{ nmol cm}^{-1} \text{ s}^{-1} \text{ Torr}^{-1}$  for the *H. portusjacksoni* egg capsule material (Appendix 3.3, p. 196), a value twice that of Diez and Davenport (1987), five times greater than recorded by A. Gannon (1992) and 2.5-8 times higher than that recorded for teleost capsules (Rombough 1989).

The higher value of  $K_{O_2}$  in *H. portusjacksoni* egg capsules may be a consequence of the much larger embryo (hatchling size of 55 g vs. 3 g for both *S. canicula* (Diez and

Davenport 1987) and *R. erinacae* (Pelster and Bemis 1991)). Larger eggs need the additional support and protection of a thicker egg capsule but must deliver more oxygen to the larger embryo. The egg capsule of *H. portusjacksoni* is 0.1 cm thick (chapter 2, section 2.3.1, p. 19), three times thicker than *S. canicula* (0.033 cm - Diez and Davenport 1987). Thus, the greater diffusive capabilities of the *H. portusjacksoni* capsules, as evidenced by a higher  $KO_2$  value, may compensate for its relative thickness. With the  $KO_2$  value calculated in this study, approximately 8.3 mmol  $O_2$  could diffuse across the capsule over 120 days, more than enough to sustain aerobic metabolism while the egg is closed.

#### 5.4.2.3 Role of external gill filaments

There is little doubt that external gill filaments have a respiratory function (Baranes and Wendling 1981, Hamlett et al. 1985a). They possess the necessary anatomical requirements (thinned walled and vascular) and they are present during a potentially hypoxic period of development while the egg capsule is sealed. They first appear when internal levels are around 80 Torr and falling. It has been demonstrated that teleost and elasmobranch fish are able to shunt blood within the internal gill structure (Piiper and Baumgarten-Schumann 1968, Cooke 1980, Piiper and Scheid 1982, Pärt et al. 1984, Laurent and Perry 1991), suggesting blood flow regulation. Because the external gill filaments in embryonic forms are simply extensions of the internal vessels, it is possible that circulation through them can also be controlled.

The evidence from the embryos studied here suggested a lack of regulatory blood flow through the external filaments, at least after capsule opening when filament reabsorption had commenced. It would be expected that the immediate response to prolonged exposure to hypoxic and anoxic water would be an increase in the blood flow through the external gills in order to facilitate an increase in the uptake of oxygen. This was not obvious as heart rate and visual blood flow remained relatively stable throughout the experiment, even after 20 min of exposure to anoxic water. Future studies might be encouraged to examine the heart rate and blood flow through the external filaments exposed to hyperoxic water to resolve function for these structures. If blood flow is already maximum in normoxic embryos, it might be expected that no changes would be observed in response to anoxia. Instead, hyperoxic water may produce a decrease in the observed flow. It appears that early embryos (pre-stage 11) have not yet developed a

regulatory response, since their internal gills are only just becoming operative. Further experimentation is required to determine the developmental stage at which embryos develop the ability to regulate blood flow through the gills.

The embryos chosen for this investigation were from capsules which had been opened to seawater for less than one month. The filaments, which were extensive, were in the process of being reabsorbed back into the internal gills. The role as a respiratory aid is unlikely to change with time for as long as the external filaments are present, but the relative importance to the overall oxygen uptake of the embryo will vary with development. Prior to egg capsule opening, gas exchange across the external filaments is likely to be of far greater importance, because of the limited convection within the egg. After opening, during the resorption of the external filaments, oxygen uptake across these vessels is no longer critical because the internal gills are becoming functional and the egg capsule is now an open-flow system.

### **5.4.3 Ventilation**

#### **5.4.3.1 Ventilation patterns**

Ventilation patterns and their control in adult teleosts and elasmobranchs are well documented. However, similar research in embryonic forms is scarce. Among the elasmobranchs, there are several ways in which the embryo is provided with its respiratory needs. Most elasmobranchs are viviparous and oxygen requirements may be provided by a placental connection, while others bathe the embryo in an oxygen rich uterine fluid throughout development (ovoviviparity) (Wourms 1977,1981, Hamlett 1989b). In contrast, oviparous elasmobranchs are not provided with oxygen throughout development by the parent. The oviparous capsule is designed to open after a period of time and allow water to flow in through the respiratory slits, driven by the convection currents created by the embryo (Luer and Gilbert 1985). While the capsule is sealed, early stage *H. portusjacksoni* embryos are observed to be moving in a whip-like motion, presumably to stir the albumen surrounding the embryo. Similar descriptions of early embryonic activity are reported for bamboo sharks, *C. plagiosum*, swell sharks, *C. venriosum*, spotted dogfish, *S. canicula* and skates, *R. erinacae* (Thomason et al. 1996, Long and Koob 1997, Meehan et al. 1997, Tullis and Peterson 1997). Diffusion of oxygen through the egg capsule was sufficient to supply only the younger developmental stages of the skate and swell shark before the egg capsule opens (A.

Gannon 1992, Meehan et al. 1997). Older embryos were supplied with oxygen by means of convectional currents through the respiratory slits of the egg capsule created by the thrashing tail movements of the embryo. Once the capsule is open, the embryo generates currents which draw seawater through the capsule. Most oviparous elasmobranch embryos develop tail appendages which greatly assist in the movement of seawater through the capsule. The stage 11-12 *H. portusjacksoni* embryo is almost constantly moving, with its head downwards and the caudal region held towards the broadside case opening. The last 8-12 segments on the tail are toughened denticles which form a spoon-like feature similar to the ray, *R. brachyura* (Clark 1926). The tail moves in a circular "scooping" fashion bringing water into the immediate area of the embryo similar to the whip-like extension of the tail in embryonic clearnose skates, *R. eglanteria*, which aid water circulation (Luer and Gilbert 1985). A distinct tail appendage, made of muscle and notochord, can be identified early in the development of the skate, *R. erinacae*, which is subsequently lost after hatching (Long and Koob 1997). The tail appendage is placed into one of four horns of the capsule and the embryo is able to generate regular waves of bending along the tail which are amplified at the occupied horn. This tail pump mechanism actively draws water into the capsule through the unoccupied horns. The swell shark capsule is also actively ventilated by embryonic currents driven by the tail, but it can also be ventilated passively (Meehan et al. 1997). If the capsule is placed into a current of 0.2 knots, water pressure differences across the capsule will cause water to flow into the capsule at no cost to the embryo.

As the embryo grows larger, the tail assisted currents are no longer possible because of spatial limitations within the capsule. Between stages 13 and 15, *H. portusjacksoni* is positioned in the capsule such that it is almost doubled over on itself with the tail folded over the top of the head (Figure 5.7, p.150). The embryo now uses oropharyngeal ventilation by placing its snout in the narrow end of the capsule where it forms a tight seal. Water is then pumped into the capsule and over the gills by active muscular gill movements for the remaining of the incubation period.

#### **5.4.3.2 Ventilation flow rate**

Two types of ventilation have been measured in this study: capsule ventilation and gill ventilation. Capsule ventilation refers to the flow of water through the whole capsule driven by movements of the stage 12 embryo. Only a proportion of the water will flow

through the gills of the embryo inside. The results from the present study show that stage 12 *H. portusjacksoni* embryos ventilate their egg capsules at an average  $12.2 \text{ ml min}^{-1}$  under normoxic conditions at  $20^{\circ}\text{C}$ . Gill ventilation occurs when the stage 13-14 embryos place their head into the narrow end of the capsule and draw water directly over the gills at  $30.0 \text{ ml min}^{-1}$ . The lower capsule ventilation rates may be the result of possible mixing of fluids in the egg capsule before the water with the dye is expelled, therefore underestimating the ventilation rate measured. Assuming that the ventilation rate scales with body mass to the same degree as metabolic rate in elasmobranchs (0.74 Pritchard 1958), the mass-specific gill ventilation flow rate is similar to other studies which measured gill ventilation on juvenile and adult elasmobranchs (Table 5.8). The effect of temperature on gill ventilation parameters is significant (Watters and Smith 1973, Campagna and Cech 1981, Steffensen et al. 1984) and should be considered when comparing data. Comparisons are made easier by correcting data to a single temperature, but problems arise because the relationship between ventilation rate and temperature has not been assessed in elasmobranchs. I have used the value for  $Q_{10}$  for metabolism of 2.2, generalised for elasmobranchs by Brett and Blackburn (1978), to compare data. Where mean body mass is unknown, I have calculated a mean value from the cited mass range. Inherent difficulties arise when attempting to compare ventilation results from different studies. The nature of variation among experimental techniques invariably induces differences in results, even in the same species. From Table 5.8, it can be seen that ventilation flow rates (at  $20^{\circ}\text{C}$ ) vary from 122-190  $\text{ml min}^{-1}\text{kg}^{-0.74}$  for *S. stellaris*, while differences are even greater in *S. canicula* (822-1332  $\text{ml min}^{-1}\text{kg}^{-0.74}$ ).

#### **5.4.3.3 Effect of hypoxia on ventilation**

It is well documented that the initial response to hypoxia in adult fish is a marked increase in ventilation, in order to facilitate a greater uptake of the available oxygen (Watters and Smith 1973, Lomholt and Johansen 1979, Campagna and Cech 1981, Steffensen et al. 1984). The ventilatory response to hypoxia is a combination of two variables ; increased ventilation by stroke volume and frequency. Increased ventilatory flows maintain a higher  $\text{PO}_2$  of the water and reduce the boundary layer at the respiratory surface. As a consequence, the extraction coefficient of oxygen, that is, the ratio of the amount of oxygen used to the amount of oxygen ventilated is reduced (Hughes 1966, Dejourns 1975).

species	range of body masses (kg)	ventilation flow rate (ml min <sup>-1</sup> )	mass specific ventilation flow rate (ml min <sup>-1</sup> kg <sup>-0.74</sup> )	T°C	corrected ventilation flow rate (20°C) (ml min <sup>-1</sup> kg <sup>-0.74</sup> )	source
<i>Scyliorhinus stellaris</i>	1.8-3.2	195	99	16	135	Baumgarten-Schumann & Piiper 1968
<i>Scyliorhinus stellaris</i>	0.97-2.5	240	159	19	172	Randall et al. 1976
<i>Scyliorhinus stellaris</i>	2.0-2.9 (mean 2.6)	320	158	16-19	192	Piiper et al. 1977
<i>Scyliorhinus stellaris</i>	1.75-3.6 (mean 2.5)	197	100	17	127	Heisler et al. 1988
<i>Scyliorhinus canicula</i>	0.15-0.2	120	436	12	819	Hughes & Umezawa 1968
<i>Scyliorhinus canicula</i>	0.8	760	894	15	1326	Short et al. 1979
<i>Raja ocellata</i>	0.75-5.0	150	68	12	129	Graham et al. 1990
<i>Squalus acanthias</i>	1.3-4.8 (mean 2.5)	433	220	11-13	413	Millen et al. 1966
<i>Squalus suckleyi</i>	2.5-6	500	171	11	348	Lenfant & Johansen 1966
<i>Heterodontus portusjacksoni</i>	0.013-0.043	22	353	20	353	this study

Table 5.8 Ventilation flow rates (ml min<sup>-1</sup>kg<sup>-0.74</sup>) for elasmobranchs, at experimental temperatures and corrected to 20°C assuming a Q<sub>10</sub> of 2.2. Where a mass or a mean mass was not given, I have used the mean from the ranges provided.

The results from this study show no significant difference in the ventilation flow rate during normoxic and hypoxic exposure. In addition, there was no significant response of ventilation frequency with declining PO<sub>2</sub>, even to levels of 15-20 Torr. The apparent lack of ventilatory regulation may be because once the capsule is open, the water entering the capsule is always saturated (Appendix 1, Fig.7, p. 187), and there is no need to regulate ventilation because the embryo is not exposed to variable PO<sub>2</sub>. Because of this, regulatory mechanisms may not have developed yet. Similarly, Thomason et al. (1996) demonstrated severe hypoxia (down to 0.1% oxygen) over several hours produced no change in ventilatory frequency in embryos, as recorded as tail beats per minute (in embryos in closed capsules and with external gill filaments) and as buccal movements (in larger embryos that do not use body movements). Diez and Davenport (1987) also reported no change in ventilation frequency in *S. canicula* until very low levels of ambient O<sub>2</sub> (30 Torr), at which point the ventilation frequency dropped dramatically.

There have been several studies examining the ventilatory response to hypoxia on adult *S. canicula*. No change in ventilation frequency with progressive hypoxia was reported by Butler and Taylor (1971,1975a) and Short et al. (1979). The lack of ventilatory response in these studies may be due to experimental conditions already producing maximal responses in so-called resting animals, the responsible variable not being measured, or a true result indicating no ventilatory response. For example, the animals used by Butler and Taylor (1971) were agitated in the experimental chambers, thereby increasing the recorded cardiac and ventilatory response above resting values. A significant increase in the ventilation frequency (49%) to hypoxia was recorded in the same species, not subjected to surgical operation or physical restraint (Hughes and Umezama 1968, Metcalfe and Butler 1984), indicating the importance of minimal stress on results. An increase in stroke volume was reported in hypoxic *S. canicula* by Short et al. (1979), but unfortunately neither Butler and Taylor (1971, 1975a) or Metcalfe and Butler (1984) assessed this variable in their experiments, although the latter study did observe greatly increased ventilatory movements in response to hypoxia.

The ventilatory response to hypoxia has been investigated in several teleost species. Stable oxygen consumption rates in response to hypoxia (50 Torr) are maintained by increasing the ventilatory stroke volume and ease of oxygen transfer across the respiratory surface in the Starry flounder, *Platichthys stellatus* (Watters and Smith 1973). Ventilatory stroke volume in undisturbed, resting flounder, *P. flesus*, doubled in response to acute hypoxic exposure, while the percentage of oxygen extracted at the gills decreased by 20% (Kerstens et al. 1979). A similar increase in ventilatory stroke volume was observed in response to severe hypoxia (40 Torr) in the Sacramento blackfish, *Orthodon microlepidotus* (Campagna and Cech 1981). However, there was no corresponding change in the percentage of oxygen extracted or in the frequency of gill ventilation.

## 5.5 SUMMARY

Early metabolism from embryos in closed egg capsules was determined using several techniques. Theoretical calculations showed that there was not enough oxygen in the egg at the time of laying to allow the embryo to utilise aerobic metabolism during the 120 day period prior to egg opening. For this to occur, oxygen must diffuse through the egg capsule. Diffusion coefficients calculated in the present study indicate that diffusion from the environment can supply the embryo with enough oxygen at least in the early stages of development. Young embryos (less than stage 10) are seen to be constantly thrashing around in an attempt to disrupt the layer of albumen that surrounds them and enhance oxygen transfer between the albumen and themselves. Diffusion through the capsule may become limiting in the older embryos, leading to the capsule opening, thus eliminating the restrictions in oxygen transport to the embryo imposed by the capsule. In the closed capsule, as the embryo grew, the metabolic rate was matched by an increase in the gradient of  $PO_2$  as defined in the Diffusion Equation.

Once the capsule opened, closed and open respirometry were employed to measure oxygen consumption in embryos stage 12 onwards. Statistical analysis demonstrated no significant difference in these two methods, allowing the combination of open and closed results to strengthen the analysis. The relationship between mass and oxygen consumption was defined by logarithmic equations on log-log transformed data. Mass exponents (0.22 - 0.83) were comparable to those cited for other species in the literature. However, the relevance of mass exponents as comparative tools is questioned, with a discussion on the failure of most studies to meet all of the necessary requirements of analysis.

The effect of hypoxia and the evaluation of a critical  $PO_2$  was investigated in stage 12 embryos between 9 and 14 g. These embryos behave as metabolic regulators at  $PO_2$ 's above 70 Torr. Below 60-70 Torr (the critical  $PO_2$  or  $P_c$ ), the oxygen consumption begins to fall with ambient oxygen levels. Measurements of the  $PO_2$  of the albumen from sealed eggs show that stage 10 embryos experience oxygen levels below the  $P_c$ , suggesting that younger embryos are more tolerant to low oxygen than older ones.

As ambient temperature rose, the rate of oxygen consumption of embryos increased.  $Q_{10}$  values, calculated from oxygen consumption of embryos of standard masses, decreased as the embryos aged (5g -  $Q_{10}=7.0$ , 50g -  $Q_{10}=0.95$ ). This supports the decreased sensitivity to temperature changes in older embryos reported by other workers. The high  $Q_{10}$  of younger embryos is attributed to greater activity at higher temperatures.

Ventilation through the capsule was facilitated by embryonic movement. Stage 11-12 embryos create currents with a scooping motion of their tail that draws water in and out of the capsule through the broad end respiratory slits. As the embryo grows (stage 13 to hatch), the internal volume of the capsule restricts this form of capsule ventilation. The embryo now places its snout in the narrow end of the capsule and draws water in and over the gills and out through the broad end slit. Ventilation rates of the capsule were  $12.2 \text{ ml min}^{-1}$  in stage 12 embryos, while gill ventilation in older stages was  $30.0 \text{ ml min}^{-1}$ . Hypoxia did not effect ventilation rates or frequency at any temperature, however frequency increased with temperature. Ventilation and metabolic rate were matched throughout the remaining development to maintain the capsule  $\text{PO}_2$  above 100 Torr.

## Chapter 6 CONCLUSION

*Heterodontus portusjacksoni* is an oviparous shark that lays up to 16 eggs over a 2-3 month breeding season. Eggs are laid in pairs and deposited near or within crevices in a rocky reef system during September through to November each year. Each egg contained one embryo which was incubated over 10-12 months. Eggs varied markedly in both length and mass. The mean length of an egg capsule was 128.6 mm (range 105-180 mm, n=21), while the mean total mass was 155.5 g (range 90.1-208.1, n=138).

When first laid, the egg contained a large yolk (mean mass 38.6 g) which is not directly attached to the egg capsule. The yolk was surrounded by a thin, viscous albumen, that makes up approximately 10% of the total mass. This was surrounded by a thicker albumen that originated at the respiratory slits either end of the egg capsule and coated the inside of the capsule. The thick albumen at the respiratory slits functioned to seal the egg capsule during the first 120 days of incubation. The albumen 'plugs' eventually dissolved and the egg capsule became open to the external sea water.

The embryo developed from the yolk with no nutritional benefit derived from the albumen. It is likely that respiration in the first 120 days was, as least in part, aerobic with gas exchange facilitated by an increase in the respiratory surface area through the development of vascular fins, yolk and external gill filaments. However, as the embryo developed, this is likely to become limiting thus leading to the opening of the capsule. The opening of the capsule coincided with maximal growth of external gill filament which were then quickly resorbed because they were no longer required. The external gill filaments were delicate structures which may become damaged if they remained. The internal gills were now functional, providing the mechanism for respiratory exchange, with sea water being flushed through the egg capsule by vigorous embryonic movements.

Prior to opening (40% of incubation), the embryo accumulated less than 2% of final hatchling mass. At opening, the aerobic capability of the embryo was markedly enhanced, allowing for more energy to be available for growth. This was demonstrated by the dramatic increase in embryonic mass at this time. Closely associated with this was an increase in the efficiency with which energy was transferred from the external yolk to the embryonic tissue. Growth increased markedly until about 60 % of incubation at which time it began to plateau. The sigmoidal pattern of growth was best described by logistic equations. The external yolk mass was depleted in a reverse

sigmoidal pattern. The external yolk was used directly by the developing embryo and also stored in an internal yolk sac. Internalisation of the yolk was probably a protective response to the possibility of premature hatching. In such an event, any yolk that is attached in an external sac is in danger of being damaged, resulting in embryonic death.

The *H. portusjacksoni* embryo was extremely efficient at converting available nutrients and energy into animal tissue. At the capsule opening, the aerobic capability of the embryo was markedly enhanced, allowing for more energy to be available for growth. This is seen by the dramatic increase in embryonic mass at this time. Closely associated with this was an increase in the efficiency with which energy is transferred from the external yolk to the embryonic tissue. At hatch, 78-85% of the mass of the yolk and 72-78% of the initial energy of the yolk has been transformed into the hatchling. For significantly longer incubation periods, there was no significant difference in efficiency of either mass or energy transfer to the hatchling, although a decreasing trend for both GEG and GPE was recorded at lower temperatures. The concept of longer incubation at lower temperatures leading to increased maintenance costs was supported in this study.

*H. portusjacksoni* was remarkably tolerant of adverse respiration conditions, particularly in the first 120 days of incubation. While the egg capsule was sealed from seawater, the internal PO<sub>2</sub> directly above a stage 10 embryo fell to a low of 29 Torr (approximately 20% of normoxic level). By thrashing its tail within the thin albumen, stagnant layers that may form around the embryo are disturbed and respiratory gas exchange is enhanced. It was found that a PO<sub>2</sub> of 70 Torr and below resulted in a significant decrease in the respiratory rate in stage 12 embryos, so it is likely that 29 Torr would constitute severe hypoxia. It is possible that earlier embryos are more hypoxia-tolerant and have lower critical PO<sub>2</sub> but this was not tested in this study because of the difficulty in keeping embryos alive after prematurely opening a capsule. While the capsule was sealed, a combination of diffusion and very low metabolic rate provided the developing embryo with adequate oxygen, evidenced by internal albumen PO<sub>2</sub> of greater than 70 Torr. By stage 10 of development, the metabolic rate had increased and diffusion through the capsule was limiting. The albumen PO<sub>2</sub> had fallen to below 50 Torr, leading to respiratory distress. This is likely to have led to the opening of the capsule to seawater to increase the aerobic capacity of the embryo. Once the capsule opened, embryonic metabolic rate and ventilation rate through the capsule are matched such that the PO<sub>2</sub> inside the capsule is maintained at 115-135 Torr. The embryo does not constantly ventilate the capsule, it uses up some of the available oxygen and when

the  $PO_2$  falls to levels approx 75-90 Torr, it flushes the capsule with an influx of normoxic water from the external environment. The  $PO_2$  inside the open flow capsule generally remained above 75 Torr, although it occasionally fell to as low as 37 Torr. However there was no apparent regulatory mechanism for ventilation which was similar at normoxia (160 Torr) and hypoxia (60 Torr). This may be because such mechanisms are not yet developed or not required in the oxygen saturated environment in which *H. portusjacksoni* eggs are normally found.

## APPENDIX 1 FIELD OBSERVATIONS AND EGG COLLECTION

### 1 INTRODUCTION

The reproductive biology of *Heterodontus portusjacksoni* has been documented by direct observation in the field and seawater aquaria by McLaughlin and O'Gower (1971). From their extensive nine year study of the population in New South Wales waters, we know that sexual maturity occurs at 11-14 years in females, while males mature earlier at 8-10 years of age. An annual breeding period lasting 2-3 months occurs only while the water temperature is below 18°C. During this period, the female lays 10-16 eggs. Females tend to have preferred sites in which to lay their eggs. Tagging information provides evidence for individual females returning to specific sites each season. This can be further supported by the fact that eggs are often found in pairs, threes, or sometimes quite numerous clusters within a small area. These clusters consist of freshly laid eggs, eggs from the previous season containing near-term embryos and empty egg capsules from past seasons. Localised nesting areas of the Japanese species, *H. japonicus* have also been found, supporting the idea of favoured nesting grounds in this genus (Smith 1942).

This study investigates the breeding of *H. portusjacksoni* in South Australian waters. Field observations and collection of newly laid egg capsules allows the breeding season to be identified and the seasonal frequency of breeding to be determined. The rates of embryonic mortality in the field and in the laboratory are compared. The characteristics of the egg capsule are examined as useful parameters in estimating the age of an egg. This is particularly useful in the early months of incubation when the egg capsule is sealed because the embryo cannot be seen.

## 2 MATERIALS AND METHODS

### 2.1 The field site

I chose two study sites based on their relative proximity to shore (250-300 m), shallow depth (less than 6 m), the presence of egg capsules on the beach and on the reefs, and previous sightings of adults in the area. The collection of eggs was made easier because the majority of collection trips were shore-based rather than from a boat. The main site was a section of Horseshoe Reef at Christies Beach, South Australia (35°11',138°14'); the second site was a reef located at Second Valley, South Australia (35°32',138°14') (Figure 1).

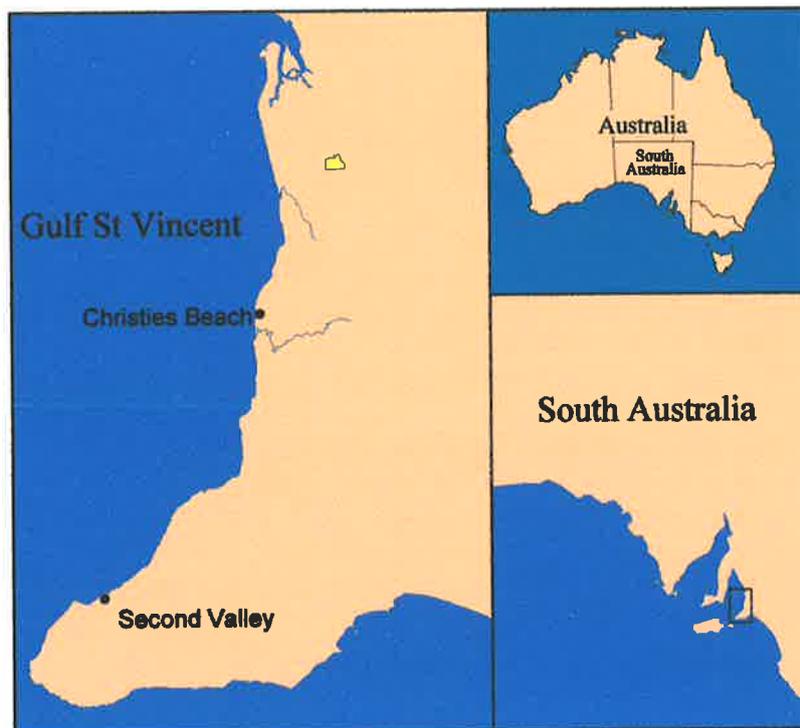


Figure 1 Site locations for egg collections in South Australia.

### 2.2 Egg collection

*H. portusjacksoni* is a nocturnal species, and egg laying presumably takes place at night, although I never witnessed the event. Therefore, in order to collect eggs as fresh as possible, I used the characteristics of the egg capsule to judge freshness (see section 4.4, p. 192). I also collected older eggs for examination. Because it was impossible to search the entire reef systems thoroughly, I selected a study area (7 m<sup>2</sup>) within Horseshoe reef. The area was defined by a 4 mm stainless steel chain held in place with 20 cm stainless steel pegs. Initially, all visible eggs (new and old) were removed from

the study site and replaced elsewhere on the reef. The area was checked for the appearance of new eggs approximately every 7-14 days for 9 months using SCUBA. Other areas on Horseshoe and Second Valley reefs were searched for the presence of eggs. The physical position of each egg within the rock crevice (broad side, narrow side or sideways uppermost) was recorded before the egg was carefully removed and placed into collection bags for the duration of the dive. Once on the boat or back at shore, the eggs were placed into aerated seawater containers and brought back to the laboratory at the University of Adelaide.

During collection, the approximate number of non-living eggs seen on the reef in any one dive was recorded. These eggs were classified as dead (indicated by small drill holes in the egg capsule caused by predatory snails) or empty (an egg capsule from a successful hatching). I collected several of the dead eggs and examined them in the laboratory for signs of predators.

### **2.3 Oxygen and temperature measurements**

During 1989, measurements of temperature and PO<sub>2</sub>, were taken at Horseshoe reef as a recording of natural conditions. Measurements were taken within crevices in which egg capsules were found. An average temperature was recorded with a hand held alcohol-based thermometer. The oxygen levels were measured using an oxygen probe (HI8543 Oxymeter, Hanna Instruments). Calibration was performed in the laboratory before going into the field. The probe was exposed to the water, while the electrical system was placed in a waterproof housing.

### **2.4 Laboratory conditions**

Eggs were distributed among one of several incubation temperature regimes of 10, 15, 18, 20 or 22°C. The eggs were put into aquaria fitted with under-gravel filters which had been operating for 2 months previously. This allowed time for the microbiological filter system acting within the gravel to be fully functional. The aquaria were placed into constant temperature rooms where air-conditioning units maintained the air temperature such that the water temperature was at the desired level  $\pm 1^\circ\text{C}$ . A light:dark cycle of 12:12 hours was maintained. The seawater was collected from local beaches and transported to the laboratory in 500L containers. The salinity of the water in the aquaria was maintained at 38-40 g/L by the addition of distilled water to replace that lost by evaporation. Aeration was achieved with several air stones in each aquarium.

Half of the water was changed every 3-4 weeks to reduce the metabolic waste build-up, or when required (for example, if an egg died and fouled the water).

### **2.5 Mortality in the laboratory**

Because it was suspected that poor aeration caused high mortalities (average 88%) in 1988 and 1989, the collections in 1990 and 1991 were subjected to strong aeration. A rack was constructed of coarse plastic mesh (50 x 50mm), held down by lead weights, and individual egg capsules were "screwed" into the rack. An air stone was placed under each egg capsule to ensure adequate oxygenation of the egg.



### 3.2 Oviposition

I generally found eggs as individuals, but I also found them in groups as large as 15 per square metre. In these groups, eggs from previous breeding seasons (empty or still with last year's embryo) as well as the current ones were next to each other. Living eggs occurred in several orientations: broad side upright, narrow side upright or lying on their side (Figures 3, 4 and 5). Observations between collections varied but generally I found that in September, an average of 15% were broad side upright, while 85% of eggs were found lying on their side (Figure 6). The situation observed later in the season (October and November) showed that 48% of eggs were broad side upright, 44% were sideways and 8% were found narrow side upright. Fresh eggs were generally loosely positioned sideways within a crevice, or lying loose in sand at the base of the reef. Older eggs were tightly wedged into crevices, requiring forceful reverse screwing action to remove them.



Figure 3 An egg found in a crevice at Horseshoe Reef with the broad end upright (see arrow).



Figure 4 An egg found in a crevice at Horseshoe Reef with the narrow end upright (see arrow). Note the gastropod on the tip of the capsule.



Figure 5 An egg found lying on its side at Horseshoe Reef (see arrow). Again note the gastropod attached to the capsule.

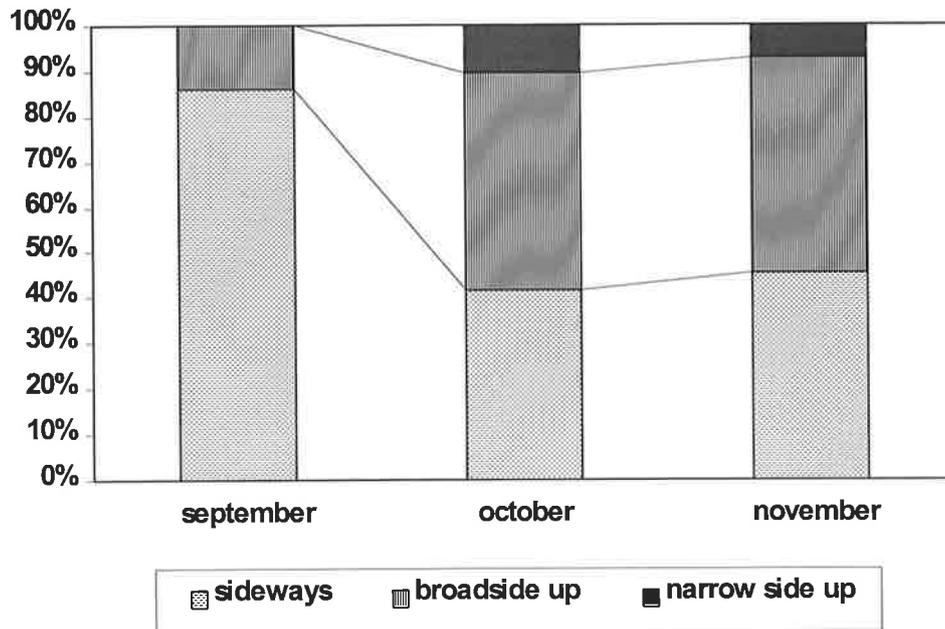


Figure 6 Percentage of fresh egg capsule position during the laying season.

### 3.3 Physical parameters

The seasonal variation of water temperature is shown in Figure 7. Water temperature varied between a low of 11.7°C in August (mid winter) to a high of 21.3°C in March (end of summer).

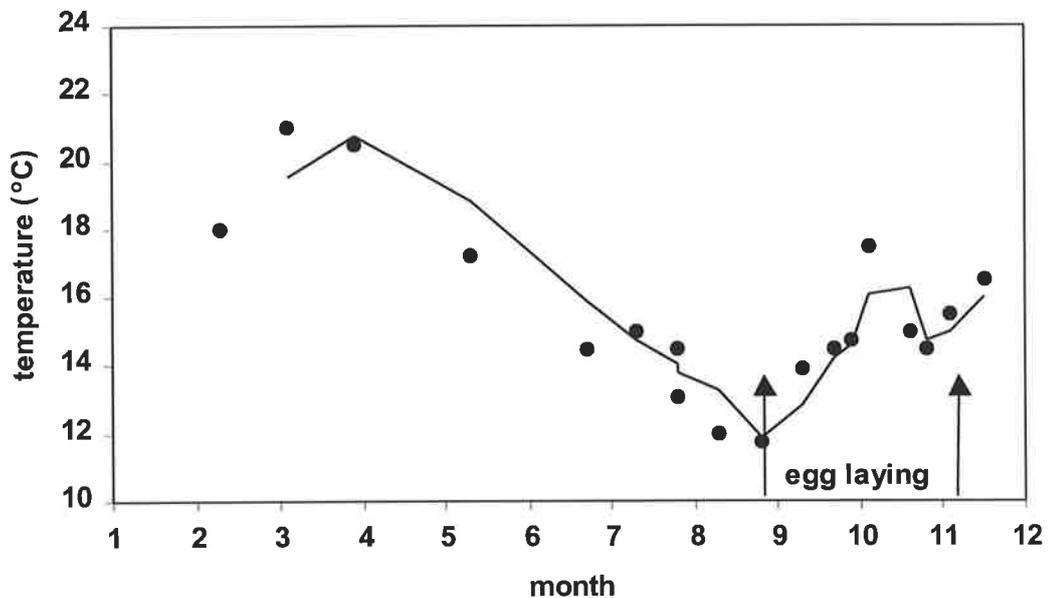


Figure 7 Temperature profile at Horseshoe Reef. Temperatures are taken directly adjacent to the egg capsule. Note egg laying takes place between August and November each year, when water temperature rises above 14°C.

At all times the oxygen levels were saturated or above saturation at depths ranging from the surface to 6 m. This was not surprising since the wave action over the reef was always vigorous, constantly aerating the water.

### 3.4 Natural mortality

Approximately 50% of the eggs that I observed in dives over the sites did not contain live embryos and were classified as non-living. These were either empty capsules (ones from which last year hatchlings have emerged) or dead. These could be easily distinguished by colour and/or by squeezing the egg capsule gently. Live eggs were uniformly green or brown, but the capsule of dead eggs developed a yellow colour in between the flanges (Figure 8). Empty capsules were of green-brown in colour. Gentle squeezing resulted in collapse of the capsule (if empty) or expulsion of a thin, yellow fluid through small openings (if dead). The yellow fluid was a mixture of yolk, albumen and possibly seawater, a result of the destruction of the yolk membrane.



Figure 8 A dead egg (see arrow) showing the yellowing of the capsule.

A large proportion of the non-living capsules had evidence of predators. An exact percentage was not recorded because I did not collect all of these capsules, but I estimate that at least half of these capsules were dead, rather than empty. I examined

some of the dead capsules in the laboratory and found obvious external signs of predator attack. There was often one (sometimes two) small holes which penetrated the thickness of the capsule wall. These holes were 1-2 mm in diameter and often located in the broad end of the capsule. In most capsules, the egg contents (yolk and albumen) were mixed into a fine yellow fluid. In several of these eggs, I found several annelid worms, polychaete worms or echinoderms inside the egg capsule. In some living egg capsules I observed presumed predator attacks as bites marks around the outer flange (Figure 9).



Figure 9 An egg capsule with regular "bite" marks around the flange.

## 4 DISCUSSION

### 4.1 Breeding season

Elasmobranch breeding cycles are not necessarily consistent between species within a genus. Where one species may have clearly annual cycles, others may have less clearly defined seasons. For example, while *H. japonicus* is known to lay eggs over a 7 month period between March and September each year (Smith 1942, Compagno 1984), the breeding season for *H. portusjacksoni* has been documented as being 3 months duration from the end of August to November each year (McLaughlin and O'Gower 1971, this study). No fresh eggs were collected during the months December through to July of the next year, indicating an annual cycle. During this period, the female may lay up to 16 egg capsules which are deposited in pairs approximately every 10 days (McLaughlin and O'Gower 1971).

### 4.2 Oviposition

The egg capsules occur in or near crevices where they become fixed for the remainder of incubation. The accumulation of capsules from many seasons in one area suggests that the female has favoured breeding sites and returns to them each year. The act of egg laying in the natural environment has not yet been witnessed and inferences concerning this event can only be based on observations on egg locality and position. McLaughlin and O'Gower (1971) believe that the female actively "screws" the egg capsules into rock crevices because they found eggs of *H. portusjacksoni* were all positioned broad side uppermost. The eggs recorded by Dean (Smith 1942) of *H. japonicus* were found mostly broad side upright (80%), but a percentage (20%) were located on their sides.

My field work showed *H. portusjacksoni* eggs in a number of positions. More commonly they were either sideways or broad end upright. However, a few were narrow end upright. The number of these latter capsules may be underestimated because they have a smaller surface visible to the diver, and therefore are less likely to be observed. Figure 4 (p. 186) demonstrates the difficulty in seeing such eggs. This leads me to suggest that the female does not actively place the eggs into crevices, but rather places them loosely into a crevice or in a suitable area near crevices and lets the wave action wedge them in tightly. This is supported by the fact that all fresh eggs are slightly, if at all, wedged into crevices, whereas older capsules are often tightly placed.

The Japanese divers employed by Bashford Dean (Smith 1942) recorded large nests of *H. japonicus* eggs embedded into crevices between rocks. These nests were described as being "largely concealed by several flat stones which the divers removed only with difficulty" (Smith 1942). These observations suggest that the female shark did not place her eggs deliberately. However, it does raise questions as to how the eggs got into crevices too narrow for the female. Such historic observations should not be ignored, but care should be taken in their interpretation. My observations in the field also raise questions as to how a female could purposefully place an egg. I found them within narrow fissures 0.5-1 m deep, where it was extremely difficult to reach them with an extended arm. The probability of a female positioning herself to lay or screw an egg in such crevices is doubtful.

#### **4.2 Natural Mortality**

Over 50% of the capsules observed on field visits were classified as non-living, a large proportion of which were dead. Holes in the capsule may be formed by drilling gastropods (although no evidence of gastropods inside the capsule has been found yet) or by starfish. After a predator has created a hole, other organisms can get in and destroy the continuity of the yolk sac and feast on the rich yolk. In those egg capsules examined, invertebrate predators such as annelid and polychaete worms or echinoderms were found inside the capsule. Based on extensive examination of elasmobranch egg capsules, Cox and Koob (1993) found that the principal predators were gastropods. Mortality in egg capsules with holes may also be attributed to an osmotic changes inside the capsule.

In addition, several of the capsules had damaged outer flanges (Fig. 9, p. 189). There appeared to be "bite marks" of equal size and equally spaced around the upper flanges. Divers have reported seeing adult *H. portusjacksoni* carrying the eggs in their mouth (McLaughlin and O'Gower 1971), but the form and arrangement of teeth from an adult *H. portusjacksoni* jaw did not correlate with the observed damage. It is probable that they were the result of some other predator trying to bite the egg. The shape of the egg capsule makes gaining a good mouth hold on it difficult as it would tend to project the capsule in a spiral fashion through the water when bitten (Smith 1942).

### 4.3 Mortality in the laboratory

The eggs collected early in the breeding season (September to October) may have died because of the shock of removal from the crevices in the reef, although this was done as carefully as possible. The yolk membrane of these early eggs is quite fragile (as was discovered when examining early embryos for morphological studies - Chapter 2) and is disrupted easily. Mortality of eggs in the laboratory was high in the first two years, with 88% of eggs dying. Additional aeration at the individual level during the last year experiments, appeared to lower the mortality rate to 33.3% although this was not a controlled experiment. My aim was simply to reduce the number of eggs that died. It was also possible that the age of eggs when collected had an effect on subsequent survival. The likelihood of survival to hatch was extended in those eggs collected later in the season, that is, in older eggs in which there has already been some development. Data from 39 hatchlings showed that 82% came from eggs collected late in the season (after 17 November). In the older eggs, the yolk membrane is more robust and can withstand a rougher treatment.

### 4.4 Ageing of egg

Unless the laying of an egg was witnessed, the actual age at any time subsequent to laying is impossible to know. *Heterodontus* is nocturnal by nature and therefore witnessing this event is a difficult task. Several night dives on the site proved unsuccessful in this respect.

I attempted to establish the age of freshly laid eggs by marking an area on the reef, clearing it of old capsules, and monitoring the appearance of new eggs. The date of laying of new eggs occurred sometime between visits to the site. However only nine fresh eggs were found within the marked area, while many more were found outside the area. An alternative approximation of age was determined by the characteristics of the individual egg capsule (Smith 1942, McLaughlin and O'Gower 1971). Freshly laid egg capsules were clean, soft and pliable. They were covered in mucous which probably assisted the evacuation from the female's body. The colour of a fresh capsule was olive green. The egg capsule retained the outer mucous covering for one month, gradually becoming less slimy over this period until it acquired a smooth, elastic surface. The egg capsule remained pliable for another 2 weeks, before becoming brittle. The colour changed from olive green to a dark brown after 3-5 weeks (McLaughlin and O'Gower

1971, this study). Darkening of the elasmobranch egg capsule was also reported in cat sharks, *S. canicula* (Castro et al. 1988) and skates (Cox and Koobs 1993). The degree of mucous covering, colour, softness and external growth, can indicate an approximate age with an accuracy of about 2-4 weeks. Given that the incubation period was up to 12 months, this level of accuracy was acceptable in this study.

## APPENDIX 2

### Calculation of the internal PO<sub>2</sub> of *S. canicula* eggs (from Diez and Davenport 1987 data).

For comparison purposes, I calculated the PO<sub>2</sub> inside the capsule of the oviparous embryo, *S. canicula*, immediately before opening to determine if it was sufficient to meet the metabolic demands of the embryo at this time. For this, I used data from Diez and Davenport (1987).

Rearrangement of Fick diffusion equation, I solve for ΔPO<sub>2</sub>.

$$\Delta\text{PO}_2 = \frac{\dot{V}\text{O}_2}{\text{KO}_2} \times \frac{L}{A}$$

where  $\dot{V}\text{O}_2$  = rate of oxygen consumption (nmol s<sup>-1</sup>)

KO<sub>2</sub> = the diffusion constant (nmol cm<sup>-1</sup>s<sup>-1</sup>Torr<sup>-1</sup>)

A = the total surface area of the egg capsule (cm<sup>2</sup>)

L = the thickness of the egg capsule (cm)

ΔPO<sub>2</sub> = the PO<sub>2</sub> difference across the egg capsule (Torr).

Values for the variables were taken at day 147 around which time capsule opening is said to occur.

$$\dot{V}\text{O}_2 = 0.409 \text{ nmol s}^{-1}$$

$$\text{KO}_2 = 4.935 \times 10^{-6} \text{ nmol cm}^{-1} \text{ s}^{-1} \text{ Torr}^{-1}$$

$$A = 23 \text{ cm}^2$$

$$L = 0.033 \text{ cm}$$

Solving the above equation, at the time of capsule opening, it is estimated that the ΔPO<sub>2</sub> across the capsule is 119 Torr. At normoxia, PO<sub>2 out</sub> = 160 Torr, therefore PO<sub>2 in</sub> = 41 Torr, likely to be approaching severe hypoxia for the embryo and likely to depress metabolic rate of the embryo.

## APPENDIX 3

Can the *H. portusjacksoni* embryo metabolise aerobically during the period when the capsule is closed to the external environment? The possibility of an aerobic existence is examined in the following appendix. Firstly, what are the oxygen requirements of the embryo up to capsule opening? Secondly, if these are to be met, is there enough oxygen already present in the freshly laid egg or does oxygen have to diffuse in through the capsule wall. For diffusion to occur, is the calculated Kroghs diffusion coefficient through the capsule sufficient?

### 3.1 Oxygen requirements of embryos in sealed egg capsules

How much oxygen is required to produce the embryo up until the time of capsule opening?

From Table 4.5 (p. 109) the metabolic cost of development at 20°C is 16.44 mmol O<sub>2</sub> per g of dry mass, although the assumption is made that the cost of development is constant throughout incubation. The mass of a stage 10 embryo at 20°C and at the time of egg capsule opening was 0.710 g (wet) and 0.082 g (dry) (Table 2.4, p. 23, eq. 4.5, p. 103).

Therefore to complete development up to stage 10,  $16.44 \times 0.082 = 1.35$  mmol O<sub>2</sub> is required.

### 3.2 How much oxygen is available in the fresh egg?

When the egg is first laid, there is a certain amount of oxygen present in the egg components. Here I estimate the oxygen content of the fresh egg to see if it is enough to sustain the embryo until capsule opening.

A fresh egg has an average yolk of 38.6 g (wet mass), (Table 2.1, p. 21) of which 41.3% is water (average value from Table 4.1, p. 103). Therefore the water component of a fresh yolk is 15.9 g. This is equivalent of 15.6 ml (1 ml seawater = 1.025 g). For the purposes of this calculation, the remaining yolk component is assumed to be lipid (22.7 g = 22.7 ml). The average mass of albumen in a fresh egg is 70.7 g (= 70.0 ml), most of which is water.

## Assumptions

1. The average saturation of the albumen (thick plus thin) is 80% or 125 Torr (Table 5.3, p. 148) and that the yolk is similarly saturated,
2. The capacitance of oxygen at 20°C in seawater ( $\beta$ ) = 1.54  $\mu\text{mol O}_2 \text{L}^{-1}\text{Torr}^{-1}$  (Dejours 1975),
3. Given that I could find no reference to lipid  $\text{O}_2$  capacitance, that a maximum value of 6.5  $\mu\text{mol O}_2 \text{L}^{-1}\text{Torr}^{-1}$  (being for olive oil at 37°C - Dejours 1975) be applied to yolk lipid.

The oxygen content in the yolk water is determined from the following equation:

$$\begin{aligned}\text{Oxygen content} &= \text{volume of water} \times \beta \times \text{saturation} \\ &= 0.016 \times 1.54 \times 125 = 3.08 \mu\text{mol O}_2 \text{ or } 0.003 \text{ mmol O}_2.\end{aligned}$$

Similarly, the oxygen content in the yolk lipids is,

$$\text{Oxygen content} = 0.023 \times 6.5 \times 125 = 18.69 \mu\text{mol O}_2 \text{ or } 0.019 \text{ mmol O}_2.$$

so, total oxygen content in the yolk is 0.022 mmol  $\text{O}_2$ .

$$\begin{aligned}\text{The oxygen content in the albumen} &= 0.07 \times 1.54 \times 125 \\ &= 13.45 \mu\text{mol O}_2 \text{ or } 0.013 \text{ mmol O}_2.\end{aligned}$$

Thus the total oxygen content in a fresh egg is 0.035 mmol  $\text{O}_2$ .

From Appendix 3.1, 1.35 mmol  $\text{O}_2$  are required and therefore there is not enough in the fresh egg to sustain aerobic metabolism through egg capsule closure.

### 3.3 Estimation of $\text{KO}_2$ for *H. portusjacksoni* egg capsules

The ease at which oxygen passes through a given material is described by Krogh's coefficient ( $\text{KO}_2$ ). This has been measured in two elasmobranch egg capsules to date. Recorded values of  $\text{KO}_2$  are  $4.93 \times 10^{-6} \text{ nmol cm}^{-1}\text{s}^{-1}\text{Torr}^{-1}$  (Diez and Davenport 1987) and  $1.779 \times 10^{-6} \text{ nmol cm}^{-1}\text{s}^{-1}\text{Torr}^{-1}$  (A. Gannon 1992).

A  $K_{O_2}$  value was calculated from data in Diez and Davenport (1987) using the equation:

$$K_{O_2} = \beta \times D_{O_2}$$

where  $\beta$  is the capacitance of seawater to hold oxygen (Dejours 1975) (at 15°C,  $\beta = 1.67 \mu\text{mol O}_2 \text{ cm}^{-3} \text{ Torr}^{-1}$ ), and  $D_{O_2}$  is the oxygen diffusivity in capsule material ( $\text{cm}^2 \text{ s}^{-1}$ ).

I estimate the value for  $K_{O_2}$  in *H. portusjacksoni* capsules using the equation below.

$$\dot{V}_{O_2} = K_{O_2} \times A/L \times (\Delta P_{O_2})$$

where  $\dot{V}_{O_2}$  = rate of oxygen consumption (nmol/s), calculated from equation 5.9

$K_{O_2}$  = the diffusion constant (nmol/cm.s.Torr)

$A$  = the total surface area of the egg capsule (minus flanges) ( $\text{cm}^2$ )

$L$  = the thickness of the egg capsule (0.102 cm) (Chapter 2, section 2.3.1, p.19)

$\Delta P_{O_2}$  = the difference in  $P_{O_2}$  across the egg capsule (Torr), taken from albumen oxygen measurements (Table 5.3, p. 148)

rearranging the equation to solve for  $K_{O_2}$

$$K_{O_2} = \dot{V}_{O_2} \times L/A \times 1/(\Delta P_{O_2})$$

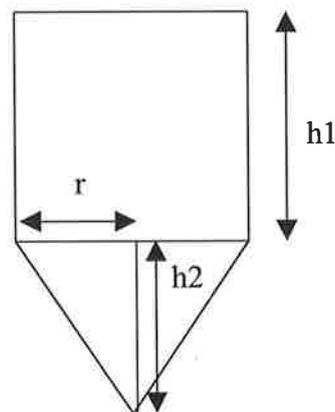
$A$  was calculated from the following equation assuming the geometric shape of the capsule as shown.

$$\begin{aligned} A &= \pi r^2 + 2\pi r h_1 + \pi r \sqrt{(h_2^2 + r^2)} \\ &= 167.7 \text{ cm}^2 \end{aligned}$$

where  $r = 2.25 \text{ cm}$

$h_1 = 8 \text{ cm}$

$h_2 = 5 \text{ cm}$



From the table below, it can be seen that the mean  $KO_2$  of  $9.69 \times 10^{-6} \text{ nmol cm}^{-1}\text{s}^{-1}\text{Torr}^{-1} \pm 1.23 \times 10^{-6}$  (9) for *H. portusjacksoni* was 2 times greater than *S. canicula* egg capsules ( $4.93 \times 10^{-6} \text{ nmol cm}^{-1}\text{s}^{-1}\text{Torr}^{-1}$  Diez and Davenport 1987), and 5.5 times greater than *R. erinacea* egg capsules ( $1.779 \times 10^{-6} \text{ nmol cm}^{-1}\text{s}^{-1}\text{Torr}^{-1}$  A. Gannon 1992).

stage of development	embryo length (mm)	embryo wet mass (g)	$\dot{V}O_2$ (nmol.s <sup>-1</sup> )	$\Delta PO_2$ (Torr)	$KO_2$ (nmol.cm <sup>-1</sup> s <sup>-1</sup> Torr <sup>-1</sup> )
4	8.0	0.002	0.129	11	$7.13 \times 10^{-6}$
4	8.3	0.005	0.209	7.5	$16.9 \times 10^{-6}$
5	13.8	0.006	0.229	32	$4.35 \times 10^{-6}$
5	15.5	0.021	0.439	20	$13.4 \times 10^{-6}$
7	29.5	0.100	0.988	77	$7.8 \times 10^{-6}$
7	30.0	0.111	1.043	80	$7.9 \times 10^{-6}$
9	36.5	0.207	1.442	88.5	$9.91 \times 10^{-6}$
9	37.0	0.192	1.386	90	$9.37 \times 10^{-6}$
10	43.5	0.374	1.959	114	$10.5 \times 10^{-6}$
					mean = $9.69 \times 10^{-6}$

### 3.4 Is diffusion into the egg capsule is enough to sustain aerobic metabolism in the young embryo.

Given a Krogh's value of  $9.69 \times 10^{-6} \text{ nmol cm}^{-1}\text{s}^{-1}\text{Torr}^{-1}$ , will this allow enough oxygen to sustain the embryo before the capsule opens?

The estimated total oxygen required before the egg capsule opens is between 1.35-4.3 mmol (determined by extrapolation of the consumption versus incubation data) and from calculations in appendix 3.1).

Assumption : The average partial pressure difference between the external medium and internal contents over the 120 days of closed capsule was 50 Torr.

Equation:  $1/X = 1/K_{O_2} \times 1/A \times L \times 1/t \times 1/\Delta P_{O_2}$

where X = the amount of oxygen diffusing across (nmol)

$K_{O_2}$  = diffusion coefficient =  $9.69 \times 10^{-6} \text{ nmol cm}^{-2} \text{ cm}^{-1} \text{ s}^{-1} \text{ Torr}^{-1}$

A = the total surface area ( $\text{cm}^2$ ) =  $167.7 \text{ cm}^2$

L = the thickness of membrane (cm) =  $0.102 \text{ cm}$

t = the time (s) = 120 days =  $1.0368 \times 10^7 \text{ s}$

and  $\Delta P_{O_2}$  = the difference in partial pressure (Torr)

Calculation:  $1/X = 1/9.69 \times 10^{-6} \times 1/167.7 \times 0.102 \times 1/(1.0368 \times 10^7) \times 1/50$

$X = 8.26 \times 10^6 \text{ nmol}$  or  $8.26 \text{ mmol } O_2$

The above calculation demonstrates that there is enough oxygen diffusing in to sustain aerobic metabolism during the period of egg capsule closure.

Alternatively, using A. Gannon (1992),  $K_{O_2} = 1.779 \times 10^{-6}$ , then  $X = 1.52 \times 10^6 \text{ nmol}$  or  $1.52 \text{ mmol } O_2$  or using Diez and Davenport (1987),  $K_{O_2} = 5 \times 10^{-6}$  then  $X = 4.26 \times 10^6 \text{ nmol}$  or  $4.26 \text{ mmol } O_2$

## APPENDIX 4

### Matching of ventilation and respiration

The possibility of the embryo matching ventilation and respiration such that  $PO_2$  is maintained at a constant level inside the capsule after the capsule opens is examined below.

Assumption : that equations 3.17 (p. 75) and 5.9 (p. 145) adequately describe the relationship between incubation period and whole wet mass, and between mass and oxygen consumption at 20°C.

Rearrangement of the Fick equation, to solve for  $\Delta PO_2$

$$\Delta PO_2 = 1/VR \times \dot{V}O_2 \times 1/\beta_{O_2}$$

where  $\Delta PO_2$  = the difference in oxygen partial pressure across the capsule (Torr)

VR = ventilation flow rate ( $L \text{ min}^{-1}$ )

$\dot{V}O_2$  = oxygen consumption ( $\mu\text{mol min}^{-1}$ )

$\beta_{O_2}$  = the capacitance of water to hold  $O_2$  ( $=1.54 \mu\text{mol L}^{-1}\text{Torr}^{-1}$ )

The  $PO_2$  inside an egg capsule ( $PO_{2in}$ ) (see table below) was then calculated by rearrangement of the following equation:

$$\Delta PO_2 = PO_{2out} - PO_{2in}$$

where  $PO_{2out}$  = the  $PO_2$  of external water ( $=160 \text{ Torr}$ )

Stage	mass (g)	VR (L min <sup>-1</sup> )	VO <sub>2</sub> (μmol min <sup>-1</sup> )	ΔPO <sub>2</sub> (Torr)	PO <sub>2in</sub> (Torr)
12	13.2	0.0125	0.747	38.8	121.2
12	13.3	0.006	0.75	81.2	78.8
13	14.95	0.00899	0.797	57.6	102.4
13	14.95	0.012581	0.797	41.1	118.9
13	14.95	0.021044	0.797	24.6	135.4
13	15.42	0.012032	0.81	43.7	116.2
14	39.2	0.018506	1.32	46.3	113.7
14	39.2	0.033478	1.32	25.6	134.4
14	39.2	0.021816	1.32	39.3	120.7
14	42.49	0.041043	1.371	21.6	138.3
14	42.49	0.035184	1.371	25.3	134.7

Respiratory and ventilatory variables during normoxia. Mass was calculated from known incubation periods (equation 3.17) and  $\dot{V}O_2$  was calculated from equation 5.9 (p. 145). Ventilation rates were measured directly.

From this, it can be theorised that the inside of the capsule is generally maintained at a high PO<sub>2</sub> of above 100 Torr (range 79 and 138 Torr), hence the embryo is able to match ventilation and respiration such that favourable oxygen levels are maintained.

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