EMBRYONIC DEVELOPMENT AND EARLY LIFE HISTORY OF THE SOUTHERN CALAMARY, *SEPIOTEUTHIS AUSTRALIS* QUOY & GAIMARD, 1832

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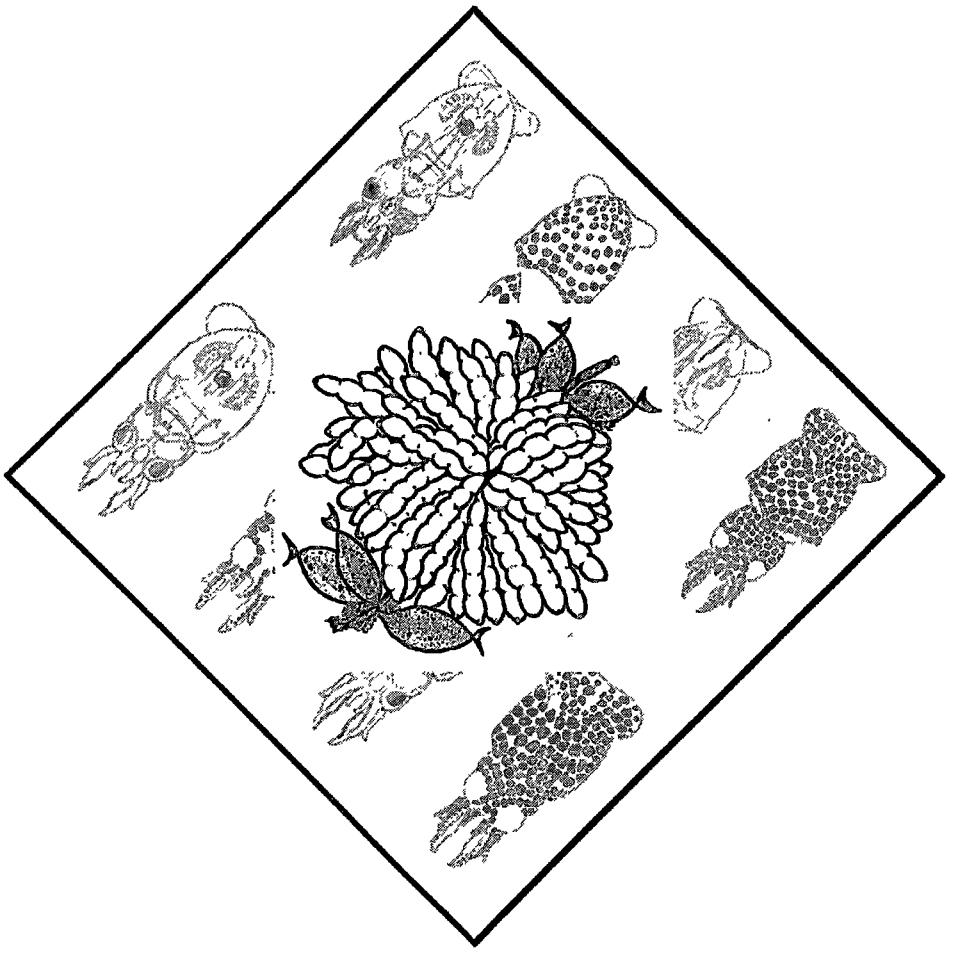

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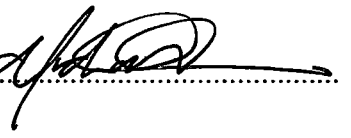
Michael Steer.....  Date.....14 FEB 2004.....

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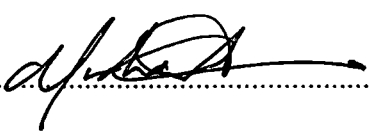
Michael Steer.....  Date...14 FEB 2004.....

ABSTRACT

The southern calamary, *Sepioteuthis australis* is a sub-annual, commercial species that exhibits little to no generation overlap. Therefore, the strength of one generation depends on the reproductive success and survivorship of offspring spawned by the previous generation. Quantitative estimates of mortality rates during embryonic developmental and the subsequent juvenile phase are virtually non-existent, therefore it is not understood what role early life history plays in determining recruitment strength. Through extensive egg surveys and laboratory experiments this project investigated the role of fluctuating environmental temperature; the position of the embryo within an aggregated egg mass; the substrate upon which its attached; the density of the egg mass; the role of fouling organisms colonising the eggs; and the effects of maternal condition on the developing embryo. Furthermore this study investigated mortality rates in the subsequent paralarval phase by using novel collection methods and statolith measurements to explore the 'bigger is better' hypothesis.

Embryo mortality rates were highly variable both spatially and temporally ranging from 2 to 25%. Dramatic increases in mortality rates were not strongly associated with natural fluctuating temperatures and there was weak evidence suggesting that fluctuating salinity was responsible. Examining embryonic development in relation to the egg mass revealed that the position of the embryo within the mass influenced hatching success. Embryos located in the centre of the mass, where egg density is greatest, developed slower and suffered higher mortality rates than those located around the periphery. This was attributed to the inability of the embryos to adequately respire and eliminated metabolic wastes and was exaggerated in large, dense egg masses that had been physically dislodged from attachment.

Maternal condition also influenced embryo mortality. Using a model, multiple spawning cephalopod, conducive to laboratory manipulation (*Euprymna tasmanica*) revealed that maternal ration and not temperature significantly effected egg viability. Low ration females produced sub-optimal eggs where 60% of embryos

failed to develop. Egg viability deteriorated over successive clutches and by the third clutch 100% of the eggs died suggesting that low ration eggs were not receiving their full complement of maternally derived resources. Embryo mortality did not exceed 60% in high ration females regardless of treatment.

Hatchling size was extremely variable ranging from 4.3 to 7.3 mm (ML), with significantly larger animals hatching out in November and the smallest in February. By comparing natal statolith dimensions between recently hatched (< 13 hrs old) and adult *S. australis* it was possible to determine whether size selective processes were operating during the early life history. In all but one month a significant difference between the size distribution of the natal radii in hatchlings and adults was found and was due to the absence of adults with small natal radii. This indicated that smaller hatchlings were less likely to recruit, suggesting that an element of size-mediated mortality exists in *S. australis* populations.

This study is the first to quantify early mortality rates and identify processes responsible in an inshore, multiple spawning cephalopod. Results obtained will aid in reducing some of the variability encompassed within existing stock-recruitment relationships, potentially improving predictive recruitment models and allowing fisheries managers to make more informed decisions about commercial squid fisheries.

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I am also appreciative for the willingness and good company of those volunteers who suffered the often dreary, cold, Tasmanian winter nights, scouring the shallows for *Euprymna.* Thank-you; Natalie Moltschaniwskyj, David Sinn, Colin Johnson, Tom Fox-Smith, Lou Ward, Belinda McGrath-Steer, Shane Roberts, Kerri Lynch, Jayson Semmens and John Forsythe. I am also extremely appreciative of the additional efforts Nat, Dave, Colin and Tom went to, in order to maintain my tank system and feed captive animals in my absence.

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CHAPTER ONE

GENERAL INTRODUCTION

1.1 POPULATION DYNAMICS

With the exception of *Nautilus* spp. cephalopods are predominantly sub-annual species and as a consequence exhibit little to no generation overlap (Boyle 1990). Therefore, the strength of one generation critically depends on the strength and spawning success of the previous generation. As a result cephalopod fisheries are composed entirely of recruits, therefore, management must aim to protect spawning stock to ensure enough eggs are laid to produce the next generation (Beddington et al. 1990; Brodziak 1998).

Although, the biology of cephalopod species is fundamentally different to the majority of teleost fish the basic ecological principles and theories that link population dynamics and fisheries management are similar; in terms of understanding and quantifying processes such as immigration, emigration, natural and fishing mortality and recruitment to estimate stock size. It is generally a 'top-down' approach where researchers have focussed on adults through commercial fishing data and scientific surveys to ascertain the health of a population or stock. This involves heavy reliance on fisheries catch per unit effort data (CPUE), a widely accepted index of abundance, which is often sparse and fragmentary (Boyle and Boletzky, 1996; Hatfield and Des Clers 1998). Nevertheless, this data is valuable in the sense that it is compiled from a comparatively large, indirect, sampling network (the commercial fishers) providing a foundation for scientists and fisheries managers to build on.

Predictive recruitment models based on spawner biomass or parental stock sizes vary in accuracy (Agnew et al. 2000) and are complicated by climatic fluctuations (Pierce and Guerra 1994). As such, it is suggested that there is no predictive understanding of the population process in cephalopods, and further research effort must investigate sources of mortality, particular those operating during the first six months of the life cycle (Hatfield and Des Clers 1998). A general lack of data on the causes of embryo and juvenile mortality and on the resulting mortality rates hampers most studies of recruitment dynamics (Boletzky 2003).

However, the importance of defining early mortality rates in cephalopods is beginning to be recognised, particularly for species of commercial interest (Cailliet and Vaughan 1983; Augustyn et al. 1994; O'Dor 1998).

Obtaining reliable, quantitative data from hatchlings and juveniles is logistically challenging, as they are largely inconspicuous (Voss 1983; but see Vecchione 1998). Assessing rates in benthic eggs, therefore, becomes an attractive first step in understanding the entire cephalopod life cycle. For inshore, neritic species (i.e. loliginids) sampling oviposited eggs is comparatively less challenging as they typically form large spawning aggregations in shallow embayments where they secure benthic eggs to the substrate (Fig 2.1, page 17). For those species that lay eggs in <20 m depth in situ observations, egg surveys, and collections are feasible via SCUBA. For other species that lay eggs in depths >20 m in situ observations are limited to expensive remote monitoring and hydroacoustic techniques (Lipinski et al. 1998). Nevertheless, for species in which spawning grounds are accessible, researchers are beginning to direct efforts to complement the 'top-down' approach with a 'bottom-up' assessment by quantifying spawning intensity and egg abundance on broader temporal and spatial scales (Moltschaniwskyj et al. 2002; Moltschaniwskyj and Pecl 2003).

Estimates of egg numbers on the spawning grounds are a valuable contribution as they can provide a relatively quick augmentative assessment of the spawning stock and potential strength of recruitment, providing there is an understanding of the species relative fecundity (Sauer et al. 1993). Estimating egg abundance alone, however, is not adequate to determine recruitment strength as the survival rates of embryos and hatchlings are unknown (Cailliet and Vaughan 1983; Scott et al. 1999). Once spawning sites have been located and densities established the next logical step would be to quantify the embryos' relative hatching success and investigate early mortality rates to fill some of these tenuous gaps.

1.2. THE 'EGG CARTON'

The loliginid egg mass represents an ideal sampling unit for experimental manipulations (Boyle et al. 2001; Boletzky 2003) and current estimates of embryo mortality are largely based on laboratory experiments. There is a clear understanding of the role of constant incubation temperatures on developmental rates and physiological boundaries (Segawa 1987; Sakurai et al. 1996; Caveriviere et al. 1996; Gowland et al. 2002a; Oosthuizen et al. 2002). What is less common are experiments examining the effects of fluctuating temperatures on the developmental process which would be more indicative of the natural environment (see Kinoshita 1982; Hoyle 2002). At present only one field study exists where embryo mortality has been assessed, with results suggesting that for Patagonian long-finned squid *Loligo gahi* mortality rates do not exceed 5% (Arkhipkin et al. 2000). Although temperature is considered the major environmental factor governing the developmental process, there is a suite of other biotic and abiotic factors that potentially perturb embryonic development and contribute to early mortality. These can be broadly categorised as (1.) environmental conditions, (2.) maternal investment, and (3) predation pressure (Fig. 1.1).

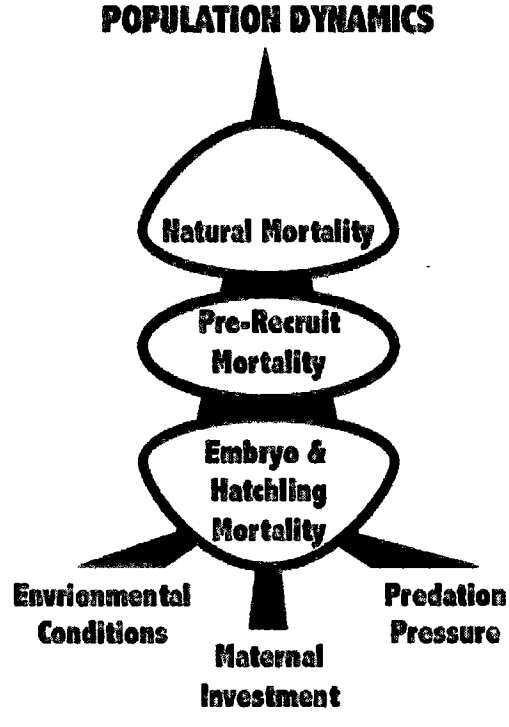


Figure 1.1. Partitioning of natural mortality into the three main contributing factors

1.2.1. Environment

Cephalopod embryos developing in benthic eggs can be considered sedentary as they are effectively anchored throughout the course of development until they individually hatch. Therefore, if mortality is a function of environmental conditions when and where eggs are laid will determine hatching success and contribute to the size of future populations (O'Dor 1998). To date the relative importance of biotic and abiotic factors on cephalopod embryonic development and survival on spawning beds has not been assessed, and laboratory experiments, manipulating conditions other than temperature, have been largely overlooked. Generally the main reason behind the collection and rearing of cephalopod eggs is to obtain healthy hatchlings for use in biomedical, growth and aquaculture studies (Forsythe and Hanlon 1988; Lee et al. 1994; Forsythe et al. 2001; Walsh et al. 2002; Vidal et al. 2002a). In most cases aquarium conditions are set to match the environment in which the eggs were collected (Boletzky and Hanlon 1983). Particular emphasis is placed upon maintaining a stable environment as fluctuations in temperature and the water chemistry (salinity, nitrates and nitrites and dissolved oxygen) can potentially perturb development, especially during early organogenesis (Choe 1966). Similarly UV light intensity and degree of agitation are monitored, as light promotes epiphytic fouling on the egg strands' surface, which may contribute to 'unsatisfactory hatching' (Choe 1966), and increased agitation promotes pre-mature hatching during the later stages of development (Boletzky and Hanlon 1983). In addition aggregated egg masses, consisting of numerous egg strands, each containing multiple egg capsules, can also constrain development, with internally located embryos effectively competing for oxygen and the elimination of nitrogenous wastes (Strathmann and Chaffee 1984). The amount of care associated with egg and embryo husbandry clearly suggests that in a dynamic fluctuating environment, characteristic of inshore spawning grounds (Augustyn et al. 1994), a degree of embryo failure is expected.

1.2.2. Maternal Investment

Embryonic development in cephalopods is direct, where embryos are totally reliant on maternally derived yolk to fuel the developmental process and hatch as

structurally and functionally competent juveniles (Jackson 1994; Boletzky 2003). Currently there is little research investigating the embryonic yolk requirements and viability of oviposited eggs, but there is evidence in the fish literature to suggest hatching success is positively correlated to maternal condition and most likely due to the quality and quantity of yolk allocation (Laine and Rajasilta 1999). There is also evidence that egg numbers and quality do not remain constant per unit biomass and must therefore be considered when attempting to forecast recruitment strength (Scott et al. 1999). The supply of food to the maturing female can also be significant in determining the reproductive potential in different fish species (Springate et al. 1985). In addition, the rate of yolk consumption in cephalopods is inversely related to incubation temperature, and hatchlings which develop in warmer temperatures need to exogenously feed earlier (Bouchaud and Galois 1990, Vidal et al. 2002). Both maternal food ration and incubation temperature can therefore provide the foundation for the developing embryo and subsequent competitiveness of the hatchling.

1.2.3. Predation Pressure

In general, benthic cephalopod eggs are extremely well protected by numerous mucous layers, and as a result there is limited evidence of egg predation in the wild (Hixon 1983; Qian and Chia 1991; Sauer and Smale 1993, Benkendorff 1999). Once the embryos hatch, however, they become more vulnerable to predation and it has been suggested that predation rates along with starvation during this early phase greatly influences recruitment strength (Okutani and Watanabe 1983). Cephalopod juveniles are difficult to quantitatively assess in the field and in many cases their basic distribution eludes researchers (Okutani and McGowan 1969; Voss 1983). This is partially due to the use of unrefined sampling techniques and equipment. In addition, little is known about hatchling and juvenile general biology as they are difficult to maintain in captivity (Laptikhovsky et al. 1993). Moreover, starvation and disease reflecting artificial rather than natural mortality factors are likely to bias mortality rates obtained from laboratory studies (Boletzky 2003). Despite these challenges, early mortality rates have been determined through planktonic surveys, in the oceanic squids, *Todarodes pacificus* (Okutani and Watanabe 1983) and *Sthenoteuthis pteropus* (Laptikhovsky et al. 1993), with both

studies concluding that mortality seems to be highest in smaller size-class squid. These studies indicate potential size-selective mortality processes operating during the squids' life history, alluding to the 'bigger is better' hypothesis.

1.3. APPLIED SIGNIFICANCE

In recent years, interest in cephalopod biology and ecology has surged due to the dramatic increase in world-wide commercial fishing pressure and the requirement of basic biological data in which to base management strategies. Catches of squid, octopus, and cuttlefish have soared since the 1950's satisfying the markets' high demand for seafood in the face of declining finfish stocks (Caddy 1983; Caddy and Rodhouse 1998; Pierce and Guerra 1994). As a consequence many commercial fishers have redistributed their effort to include cephalopods in their catch either by expanding trawling grounds, maximising cephalopod bycatch, or via directly targeting cephalopods with purpose built equipment (Rathjen 1991).

Concentration of fishing effort on spawning aggregations is a concern and a previously unmanaged practice that potentially contributed to the collapse of *Illex illecebrosus* and *Todarodes pacificus* fisheries in the North-West Atlantic and North-West Pacific Oceans, respectively (Dawe and Warren 1993). Similarly loliginid fisheries in Californian, South African and Southern Australian coastal waters are threatened by this fishing practice (Vojkovich 1998; Augustyn and Roel 1998; Moltschaniwskyj et al 2002). Temporary commercial and recreational fishing closures on known, productive spawning grounds have been enforced in South Africa, the Falkland Islands and Tasmania, aiming to protect *Loligo vulgaris reynaudii* (Roel et al. 1998), *L. gahi* (Hatfield and Des Clers 1998) and *Sepioteuthis australis* (Moltschaniwskyj et al. 2002) stocks, respectively. Effort control through closures is the most effective management tool used to regulate fishing mortality however it can potentially be a hit and miss management strategy (Roel et al. 1998). As a result, there is further requirement to advance our understanding of cephalopod biology and ecology to aid in future management decisions and therefore protecting ecologically and economically valuable resources.

1.4. THE SOUTHERN CALAMARY *SEPIOTEUTHIS AUSTRALIS*

The southern calamary, *Sepioteuthis australis* is a relatively large, robust loliginid endemic to southern Australia and northern New Zealand waters. In southern Australia it ranges from Dampier in Western Australia to Moreton Bay in Queensland, including Tasmania. For the majority of it distribution, *S. australis* inhabits coastal waters and embayments, typically associated with seagrass beds and reefs usually in depths less than 50m (Winstanley et al. 1983). It is one of the most common cephalopods in southern Australia and is a key component of the coastal ecosystem as a primary consumer of crustaceans and fishes, as well as a major food source for numerous species of fish and marine mammals (Zeidler and Norris 1989).

Southern calamary are the basis of a relatively new commercial fishery in Australia as a result of their high market value (5-15 $/kg), low set-up costs and open access to fishers with a commercial marine scalefish licence (Moltschaniwskyj et al. 2003). Mature animals predictably form large aggregations in sheltered, inshore waters during the warmer spring/summer months to spawn and are therefore an attractive, easy target for commercial fishers utilising a variety of fishing methods. Hand-jigging from small dinghies represents the preferred fishing method, however, spears and nets (dip-nets, purse seines, haul nets) are often used. In South Australia annual calamary landings peaked in 1998/1999 at 436 t worth an estimated $2 million (Triantafillos and Fowler 2000). In Tasmania annual catches also rose dramatically in 1998/1999 from historic levels generally below 20 t to 100 t valued over $0.5 million (Lyle and Hodgson 2002).

Great Oyster Bay is considered the major spawning ground for southern calamary and as such has been periodically closed to fishing during peak spawning periods (see Moltschaniwskyj et al. 2003). The unique features of this bay, including its shallow (<10m) well-protected coastline and varying influence of cool subantarctic and warm tropical water masses may make it conducive to embryonic development and post-hatching survival, potentially attracting large spawning aggregations.

Like the majority of other loliginids, *S. australis* is relatively short-lived with maximum recorded ages for females and males are 263 and 275 days respectively (Pecl 2000). During this short life span individual growth rates are rapid and highly variable (approx 4-8% day^{-1}) attaining weights of up to 3.6 kg (Lyle and Hodgson 2002). Some of this variability may be inherently a function of the individuals' thermal and nutritional environment. As a result of this 'live-fast, die-young' life style and increased fishing pressure both from the commercial and recreational fishing sectors there is concern that localised calamary populations may be increasingly susceptible to collapse.

1.5. General Objectives

The general aim of this thesis was to assess mortality rates in the early life history stages of the commercially exploited southern calamary *Sepioteuthis australis* (Cephalopoda: Loliginidae). This was achieved through field surveys and laboratory experiments with particular emphasis on identifying the roles of fluctuating temperature on natural spawning grounds; the environment and dynamics of aggregated egg masses; and maternal condition on egg viability and hatching success. Furthermore, this study investigated mortality rates in the subsequent hatchling phase by using novel collection methods and statolith measurements to explore the 'bigger is better' hypothesis.

1.6. Chapter Summaries:

This thesis consists of five data chapters, each one comprising a stand-alone manuscript, therefore there may be areas in the text that are slightly repetitive.

Chapter Two:

Embryonic Development of Southern Calamary (*Sepioteuthis australis*) within the Constraints of an Aggregated Egg Mass.

Through a series of laboratory-based experiments, this chapter's primary aim was to establish a stepwise staging criteria for the embryonic development of

Sepioteuthis australis to allow both intra- and inter-specific comparisons. In addition, the effect of egg mass density and the embryos' relative position both within individual strands and within the egg mass on the developmental process was quantified. This chapter forms the basis of a publication; *Marine and Freshwater Research* (2003 Vol. 54: 217-226).

Chapter Three:

TEMPORAL VARIABILITY IN EMBRYONIC DEVELOPMENT AND MORTALITY IN THE SOUTHERN CALAMARY (*SEPIOTEUTHIS AUSTRALIS*): A FIELD ASSESSMENT.

This field based study aimed to quantify variability in rates of development and mortality for calamary embryos throughout a spring/summer-spawning season. Due to the collective egg packaging strategy exhibited by this species, this study also described the variation in development within individual egg strands to determine if certain eggs were at a higher risk to mortality. Furthermore, the effect of biofouling on the capsule surface and its effect on mortality rates was quantified. The bulk of this study has been published in *Marine Ecology Progress Series* (2002 Vol. 243: 143-150).

Chapter Four:

FACTORS RESPONSIBLE FOR EMBRYO MORTALITY IN THE SOUTHERN CALAMARY *SEPIOTEUTHIS AUSTRALIS*: THE ROLE OF THE AGGREGATED EGG MASS.

Using results obtained from the previous chapter (three) this chapter aimed to explore mortality rates in the southern calamary on a finer resolution by focussing on the constraining properties of the egg mass. Through field and laboratory studies this study investigated the role of egg mass size, the substrate upon which it is attached, the position of the embryo within the mass and the degree of surface fouling on embryo mortality. This chapter is currently in review in *Marine Biology.*

Chapter Five:

THE ROLE OF TEMPERATURE AND MATERNAL RATION IN EMBRYO SURVIVAL: USING THE DUMPLING SQUID *EUPRYMNA TASMANICA* AS A MODEL.

Due to the logistical difficulties associated with maintaining the relatively robust *Sepioteuthis australis* in captivity this study used a model sepiolid *Euprymna*

tasmanica to explore the link between the nutritional and thermal environment in which females are exposed and the quantity and quality of the offspring produced. Previous research has examined the influence of maternal condition on fecundity and egg quality (Lewis and Choat 1993). This study takes an additional step by examining lipid bestowal over multiple clutches and quantifying hatching success. Relative proportions of polyunsaturated fatty acids (PUFA) and lipids were of particular interest, as they are considered critical for successful embryonic development (Navarro and Villanueva 2000; Boyle et al. 2001). This chapter is in press with the *Journal of Experimental Marine Biology and Ecology.*

Chapter Six:

ARE BIGGER CALAMARY (*SEPIOTEUTHIS AUSTRALIS*) HATCHLINGS MORE LIKELY TO SURVIVE?: A STUDY BASED ON STATOLITH DIMENSIONS.

Quantifying field mortality rates in an attempt to predict recruitment strength is a task that is plagued with problems and assumptions that has consistently frustrated fisheries biologists. This study aims to investigate an aspect of this problem by exploring whether 'bigger is better' in calamary stocks. Firstly, this study aimed to describe the variability in hatchling size throughout the late spring/early summer hatching season and investigated the strength of the relationship between hatchling size and a series of statolith dimensions. Using the somatic/statolith relationship this study aimed to quantify whether bigger hatchlings are more likely to survive by comparing the size frequencies of recent hatchlings with successfully recruited adults. This chapter appears in *Marine Ecology Progress Series* (2003 Vol. 261: 175-182).

CHAPTER TWO

EMBRYONIC DEVELOPMENT OF THE SOUTHERN CALAMARY (*SEPIOTEUTHIS AUSTRALIS*) WITHIN THE CONSTRAINTS OF AN AGGREGATED EGG MASS.

Steer, MA., NA Moltschaniwskyj, AR Jordan. (2003). Marine and Freshwater Research. 54: 217-226

2.1. ABSTRACT

A post-cleavage embryological scheme was established for southern calamary *Sepioteuthis australis*. Using this developmental scheme intra- and inter-specific comparisons were made. *Sepioteuthis australis* development most closely resembled that of its tropical congeneric species, *S. lessoniana* with only a few subtle heterochronies. The greatest developmental difference was observed when comparisons were made with *Loligo pealei*. These differences were attributed to developmental duration and respective egg sizes. Within *S. australis* variation in developmental rates among embryos mass was associated with the size of the egg mass, with less variation evident in smaller egg masses. Embryos located on the periphery of the egg mass and at the distal or unattached end of an individual egg strand developed significantly faster than those located deep within the egg mass. On average, embryos in small egg masses, consisting of five individual egg strands, developed significantly faster than those in dense aggregations comprised of >100 strands.

2.2. INTRODUCTION

Squid embryos are encapsulated for the duration of their development, where they are totally dependent on maternally derived yolk for nutrition, and are at the mercy of the ever-fluctuating marine environment. As a result the embryos' developmental process can be perturbed by a variety of biotic and abiotic factors manifesting themselves as slight morphological asymmetries to gross abnormalities and developmental arrest (Boyle et al. 2001). These disruptions are typically linked to unfavourable developmental conditions, namely elevated incubation temperatures (Sakurai et al. 1996), rapid or sustained changes in salinity (Palmegiano and D'Apote 1983) or extended exposure to UV-B radiation (Biermann et al. 1992). However, there is evidence of elevated mortality and developmental error for embryos developing within dense egg masses (Chaffee and Strathmann 1984). This is observed in a variety of marine species, and in each instance it is the centrally located embryos that are more likely to suffer. This is because they are effectively

crowded by neighbouring egg strands and are unable to exchange gases and waste products efficiently (Strathmann and Chaffee 1984).

Female loliginid squid typically anchor multiple egg strands to a common attachment point to form discrete egg masses. Each egg strand is comprised of multiple protective layers and can contain several hundred spirally arranged eggs (eg. *Loligo* spp) to less than 10 longitudinally aligned capsules (eg. *Sepioteuthis* spp.). Multiple spawning females often contribute to existing egg masses thereby increasing its overall density (Sauer et al. 1992). In an extreme case, *Loligo opalescens*, collectively forms egg masses up to 40 feet (12.2 metres) in diameter (McGowan 1954). However, egg masses consisting of >10 to <600 individual egg strands are commonly observed in other loliginids (eg *Sepioteuthis australis* Moltschaniwskyj and Pecl 2003). Asynchronous development and variable mortality within individual strands is evident both in laboratory reared (Ikeda et al. 1999) and field collected eggs (Gowland et al. 2002; Steer et al. 2002; Chapter three).

Cephalopods undergo direct embryonic development and to identify and define any discontinuities in the developmental process a baseline scheme must be established. Although the developmental process is continuous there has been considerable work establishing stepwise staging criteria for a number of species (Segawa et al. 1988; Baeg et al. 1992; Guerra et al. 2001). Initially the embryological process was described at equal time periods over the course of development (Naef 1928), however this staging system is considered impractical, as there is no scope to make comparisons between embryos developing under different conditions (Arnold 1990). To make this scheme practical the developmental process was modified by basing stages upon the chronological appearance of morphological structures to generate a 30 stage 'universal' scheme (Arnold 1965). Since the inception of this scheme there has been evidence of species-specific heterochronies in the appearance of morphological structures (see Guerra et al. 2001), and as a result Arnold's scheme has also been frequently modified (Segawa 1987; Baeg et al. 1992; Blackburn et al. 1998).

This study first established a stepwise embryonic developmental scheme for *Sepioteuthis australis* by incubating field-collected eggs at two temperature regimes.

This effectively sets a baseline for this species, which may benefit future embryonic comparisons, both within and between species. Using this baseline the effect of egg mass density and the embryos' relative position both within individual strands and within the egg mass on the developmental process was quantified.

2.3. Materials and Methods

2.3.1. Establishing a species specific embryological scheme

In July 2000, five recently laid, unfouled, egg masses, each comprised of >500 digitate egg strands (Fig 2.1a), were collected from shallow seagrass (*Amphibolis antarctica*) beds on the east coast of Tasmania (42°12'S, 148°17'E). Eggs were placed in two 20 L buckets filled with ambient (11°C) oxygenated seawater and transported to the University of Tasmania's aquatic facilities in Launceston. Each egg mass was divided into smaller clusters of approximately 20 egg strands and randomly placed into a series of floating baskets allocated to one of two 1400 L closed recirculating systems which were gradually increased (1°C day^{-1}) to 13°C and 16°C, respectively. Due to limited aquarium space, these two temperature regimes were chosen to tie in with a concurrent study; nevertheless, these slight daily increases in temperature were not expected to deter the developmental process. Inflow pipes and air stones were secured to the base of each floating basket, positioned directly beneath the eggs, to ensure adequate water circulation and aeration. Water quality checks were carried out three times a week and maintained at within these levels; salinity 34-36 ‰, NO_3 <10 mg/L, NO_2 <0.1 mg/L, NH_4 <0.25 mg/L. Photoperiod was set to a 12:12 hour light: dark cycle.

Five egg strands (Fig 2.1b) were randomly selected on the day of collection; thereafter five strands were collected from both temperature regimes three times a week until hatching. Each egg capsule was excised from the egg strand and examined under a stereo-microscope. Egg strand length, number of egg capsules per strand and their respective maximum length and diameter were recorded. Embryonic development was tracked by noting the appearance of morphological and anatomical structures. Embryos pre-organogenesis were observed through the egg chorion and described. Representative chilled live embryos undergoing organogenesis were

dissected from the chorion and drawn to scale with the aid of a camera lucida and digital images. A sample of pre-mature and mature hatchlings was collected from both temperature regimes, preserved in 70% ethanol and their dorsal mantle lengths measured.

The developmental process of *Sepioteuthis australis* was compared to established developmental sequences of three other loliginid species; *Sepioteuthis lessoniana* (Segawa 1987), *Loligo forbesi* (Segawa et al. 1988) and *Loligo pealei* (Arnold 1965). These three species were chosen because they share many ecological, behavioural and biological similarities with *S. australis*. Differences between species were made by comparing the order of the appearance of morphological features. Arnold's (1965) original scheme for *L. pealei* was used as a baseline and comparisons between pairs of species were completed using Kendall's coefficient of rank correlation (τ).

2.3.2. Effect of egg mass size and capsule position on embryonic development

Three large (>500 strands) recently laid egg masses were collected in August 2001 from Mercury Passage (42°40'S, 148°05'E) and transported to aquarium facilities. Each egg mass was divided into six clusters consisting of 5, 25, 50, 100, 200 and >200 strands, and each cluster was suspended by a length of nylon thread in one of six 100 L tubs connected to a closed 1400 L, 11°C recirculation system. All eggs were completely submerged during handling to reduce the risk of damage due to air exposure. Flow rates in each tub were adjusted so each egg mass was gently agitated ensuring that they were not suspended in stagnant water. Gentle surface aeration was provided and water quality was monitored three times a week. A 12:12 light: dark period was set.

Strands of embryos were harvested before hatching (~Stage 24-25) and masses containing five strands completely dissected. In the case of larger egg masses ten strands were sampled; five from the interior of the mass and five from the periphery. Embryos were dissected from each strand and staged. The position of each embryo within the strand was recorded with position 1 identifying the egg located at the fixed/proximal end of the strand progressing consecutively to the

free/distal end of the strand. Eggs that were unfertilised, undergoing abnormal development or had ceased development were scored as dead.

2.3.3. Statistical Analyses

To determine variability in embryonic development within egg strands the average developmental stage and the deviation of each embryo from the strand average was calculated (as per Steer et al. 2002; Chapter three). Differences in developmental deviation within each strand were examined as a function of the embryos relative position within the egg mass (internal/peripheral) and within the strand (distal/proximal) using a 2-way Model 1 ANOVA. Egg strands containing >7 egg capsules were not included in the analysis due to insufficient replication ($n = 3$). The effect of egg mass density on embryonic development was investigated by comparing mean embryonic developmental stages across varying egg mass densities and as a function of their respective position within the mass via a 2-way ANOVA. A Hochberg GT2 post hoc test for an unbalanced dataset was conducted to highlight significant differences among means. Egg masses containing five egg strands were not included in the analysis due to difficulties in discriminating between internally located and peripheral strands, however data was still graphically presented. In each analysis homogeneity of variances were checked via visual inspection of residual plots and no data transformations were necessary.

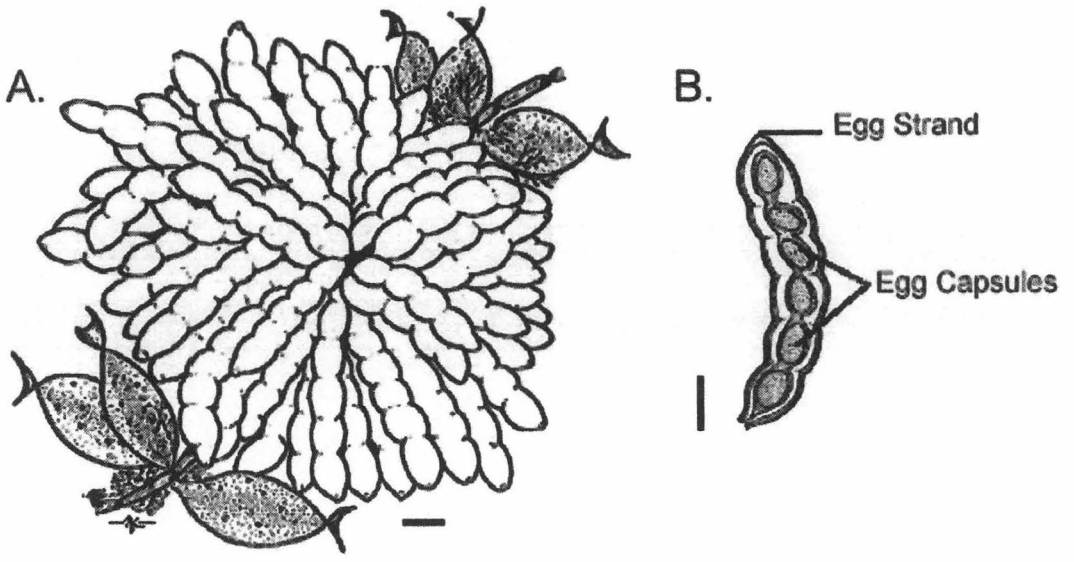


Figure 2.1. (A.) Schematic illustration of a typical *Sepioteuthis australis* egg mass composed of numerous egg strands and attached to a seagrass (*Amphibolis antarctica*) frond. (B.) An individual egg strand harbouring 6 longitudinally aligned egg capsules. (adapted from Moltschaiwskyj and Pecl (2003)). Scale bars = 10mm.

2.4. RESULTS

Sepioteuthis australis eggs are laid in white digitate strands ranging from 40 to 82mm in length and containing between 3 and 8 eggs (average 5.15, n = 215, Fig 2.1). Egg capsule volume was initially below 1.0 cm^3, however, this increased to an average of 3.7 cm^3 prior to hatching (Fig 2.2a) with no effect of temperature evident (two-tailed test, $t = 2.12$, $df = 16$, $P = 0.52$).

Developmental rate was temperature dependent with embryos incubated at 16°C beginning to hatch nine days earlier than those developing at 13°C. Duration to first hatching was 52 and 61 days, respectively (Fig. 2.2b). Hatching success was poorer in the warmer regime, with 80.5% of eggs hatching at 16°C compared with 93.0% at 13°C. Furthermore, hatchlings were significantly smaller at 16°C ($F = 23.02$, $df = 1, 273$, $P < 0.001$) on average measuring (mean ± SD) 4.80 ± 0.63 mm compared to 5.18 ± 0.62 mm at 13°C.

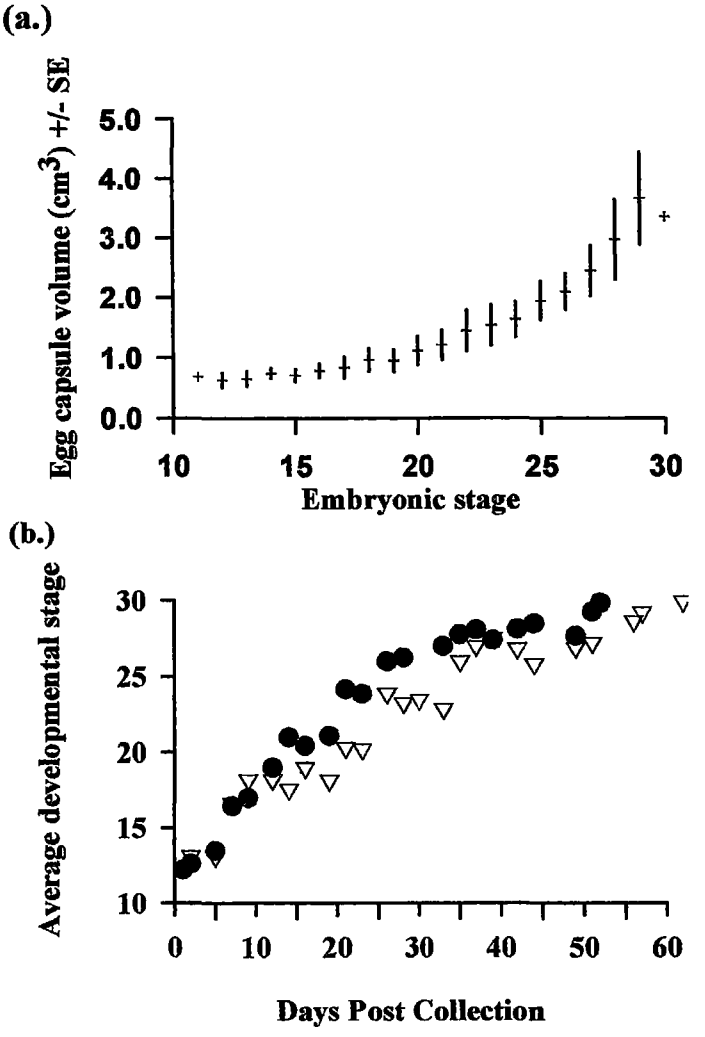


Figure 2.2. (a.) Increase in egg capsule volume throughout embryonic development of *Sepioteuthis australis*. Egg capsule volume was calculated using the formula for a uniform ovoid structure; 4/3.π. r.d, where r= the radius of the longest axis and d=the diameter of the egg capsule. (b.) Embryonic development of *Sepioteuthis australis* incubated at 13°C (∇) and 16°C (•) during the period of collection to hatching.

2.4.1. Developmental scheme

Stage 12 (Arnold 12, Segawa 10-11)(Fig 2.3)

The majority of newly collected eggs were developing through the early stages of gastrulation. At this stage the blastoderm is clearly evident at the apical end of the egg and is composed of two germ layers, consisting of ectodermic and mesodermic cells, with the former being slightly elevated from the latter. At this stage the egg capsule has already started to expand as there is a distinct separation of the capsule chorion and the yolk body.

Stage 13 (A13, S12)(Fig 2.3)

The blastoderm spreads by marginal cell divisions over the uncleaved yolk mass and at this stage covers approximately 20% of the egg.

Stage 14 (A14, S13)(Fig 2.3)

Blastoderm covers 20-40% of the egg.

Stage 15 (A15, S14)(Fig 2.3)

Blastoderm covers 40–60% of the egg.

Stage 16 (A16, S15+-16)(Fig 2.3)

Blastoderm covers 60-75% of the egg. First sign of shell gland primordia appearing as a shallow depression at the animal pole. Two slight shadows appear at either side of the embryo as a result of specialised cellulation forming the rudiments of the optic primordia. The entire embryo rotates slightly around the animal pole axis due to ciliary action on the yolk envelope (as per Boletzky 1989).

Stage 17 (A17, S16-17)(Fig 2.3)

Blastoderm covers 75-90% of the egg. Primordium of the mantle is recognised as a thickened, elevated region surrounding the shell gland. An annular ridge is evident around the optic vesicles. The areas where the arm rudiments appear begin to show faint signs of thickening. The mouth region (stomodaeum) is a crescent shaped invagination on the dorsal surface. Equatorial constriction is evident separating the future external yolk sac from the embryonic body.

Stage 18 (A17-18, S18)(Fig 2.3)

The blastoderm has nearly enveloped the entire outer yolk body. The optic vesicles support a disc-like retinal membrane whilst the mantle continues to thicken and extend, becoming more obvious. The primordia of the gills and funnel folds appear as a collar partially surrounding the mantle. The statocyst primordia are represented as distinct circular 'clouds' situated close to the outer boundary of the collar. The arm ridge becomes more pronounced and slightly separating alluding to individual arm rudiments.

Stage 19 (A18, S18+)(Fig 2.3)

The outer yolk sac is completely enveloped by the blastoderm. Gills appear and are distinguishable from the thickening posterior funnel folds. The anterior funnel folds are evident. Both the eyes and mouth begin to invaginate. Arm rudiments (A I – A V), including tentacles (T IV) become more distinct and separate buds are now prominent. The mantle continues to protrude outwards and elongate slowly enveloping the posterior shell gland. The embryo proper is compressed dorso-laterally.

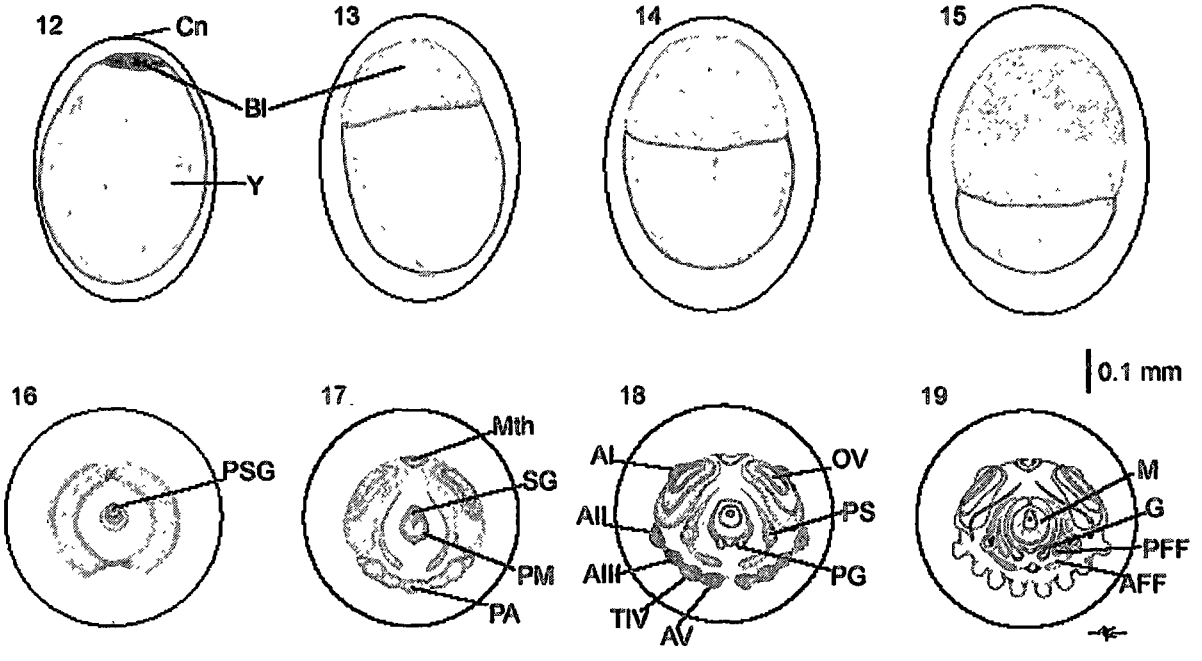


Figure 2.3. Illustrated developmental stages for *Sepioteuthis australis*. Stages 12-15 are illustrated from the lateral aspect, whereas stages 16-19 are illustrated from the apical aspect. Refer to page 31 for abbreviation key

Stage 20 (A20, S19-20)(Fig 2.4)

The shell gland is almost completely enveloped by the mantle. The eye vesicles are continuing to invaginate and beginning to close. The eyestalks are slightly protruded. Both the anterior and posterior funnel folds are thickening and converge toward the mid-line. The anal knot primordium is clearly situated between the gills.

Stage 21 (A20, S20+)(Fig 2.4)

The shell gland is completely enveloped by the mantle. Fins begin developing on the broadening mantle, which is covering approximately 10% of the gills. The optic stalks are prominent. The opening of the eye vesicle closes. The anterior funnel folds are approaching the mid-line but have not fused. The arms grow and begin to project.

Stage 22 (A21-22, S21-22)(Fig 2.4)

The mantle has continued to grow anteriorly, covering 25% of the gills. The gills are more defined and it is possible to identify slight demibranch segments. The fins are obvious and are seen as thin crescent shaped flaps on the anterior end of the dorsal mantle. The anterior funnel folds begin to fuse at the midline and thicken. The eyes are clear and display a faint pigmented annular ring. The lens primordia are visible. The eyestalks appear to be reinforced by thick ectodermic cushions (Boletzky 1988). The tips of the arms begin to splay away from the outer yolk sac and possess defined sucker bulbs. The internal yolk sac first noted.

Stage 23 (A22-23, S21-22)(Fig 2.4)

The mantle covers 50% of the gills. The posterior funnel margins approach the mid-line, whereas the anterior portion has developed into a tube. The cushioning surrounding the protruding eyestalks has thickened and extended. The retinal vesicle begins to invaginate within the iris folds. The systemic and paired branchial hearts first noted. Statoliths are evident within the now well developed statocyst. The arms continue to extend.

Stage 24 (A24, S23-24)(Fig 2.4)

Mantle completely covers the gills, anal knot and a portion of the posterior funnel margin which is now fused at the mid-line. The crescent shaped fins are also fused at the midline. The retinas display a faint pink tinge with a darker collared iris. The retinal vesicle continues to invaginate and resembles a cup-shaped dimple on the eyeball. The optic ganglia are apparent. The posterior lobes of the internal yolk sac begin to expand. The funnel is relatively well developed and is situated over a pair of distinctive, reflective statoliths.

Stage 25 (A25, S25-26)(Fig 2.4)

Mantle envelops the posterior margin of the funnel and actively contracts. Eyes increasingly pigmented from pink to red and beginning to invert. The primary lid extending from the ventral arm bases is partially enveloping the eyes and the opaque optic ganglia. An empty ink sac is evident. The branchial hearts beat asynchronously. The buccal mass is first noted.

Stage 26 (A26-27, S26-27+)(Fig 2.4)

Red chromatophores appear on the arms, head and mantle. The primary corneal lids envelop a third of the eyes, which are now pigmented dark red. The ink sac begins to fill. The anal structure is evident along with paired anal papillae. Beginning of Hoyle's organ is obvious between the fins on the dorsal mantle.

Stage 27 (A28, S27-28+)(Fig 2.4)

A second row of small brown chromatophores appears on the arms. Numerous red and brown chromatophores exist on the head and mantle. Hoyle's organ develops a T-shaped thickening. The eyes are almost completely enveloped by the transparent cornea and are faintly iridescent. The ink sac is full and functional. The embryo:external yolk sac (E:Y) ratio is 50:50. Paired tentacles begin to splay out and are obviously longer than the sessile arms. Midgut (digestive) gland first noted surrounding the internal yolksac.

Stage 28 (A28-29, S28-29+)(Fig 2.4)

Yellow chromatophores appear on the arms, head and mantle and as with the existing red and brown chromatophores are capable of firing. Eyes are completely enveloped and are highly reflective with a distinct black iris. The ink sac is densely covered with highly reflected iridophores. A dense array of suckers exists at the terminal ends of the tentacles to form the tentacular clubs. Opaque beaks are evident within the buccal mass. Internal yolk sac decreased in size whilst the caecum and midgut gland increase. E:Y ratio is 60:40 with the external yolk sac easily detached from the embryo. Premature hatching occurs if the embryo is mechanically stimulated.

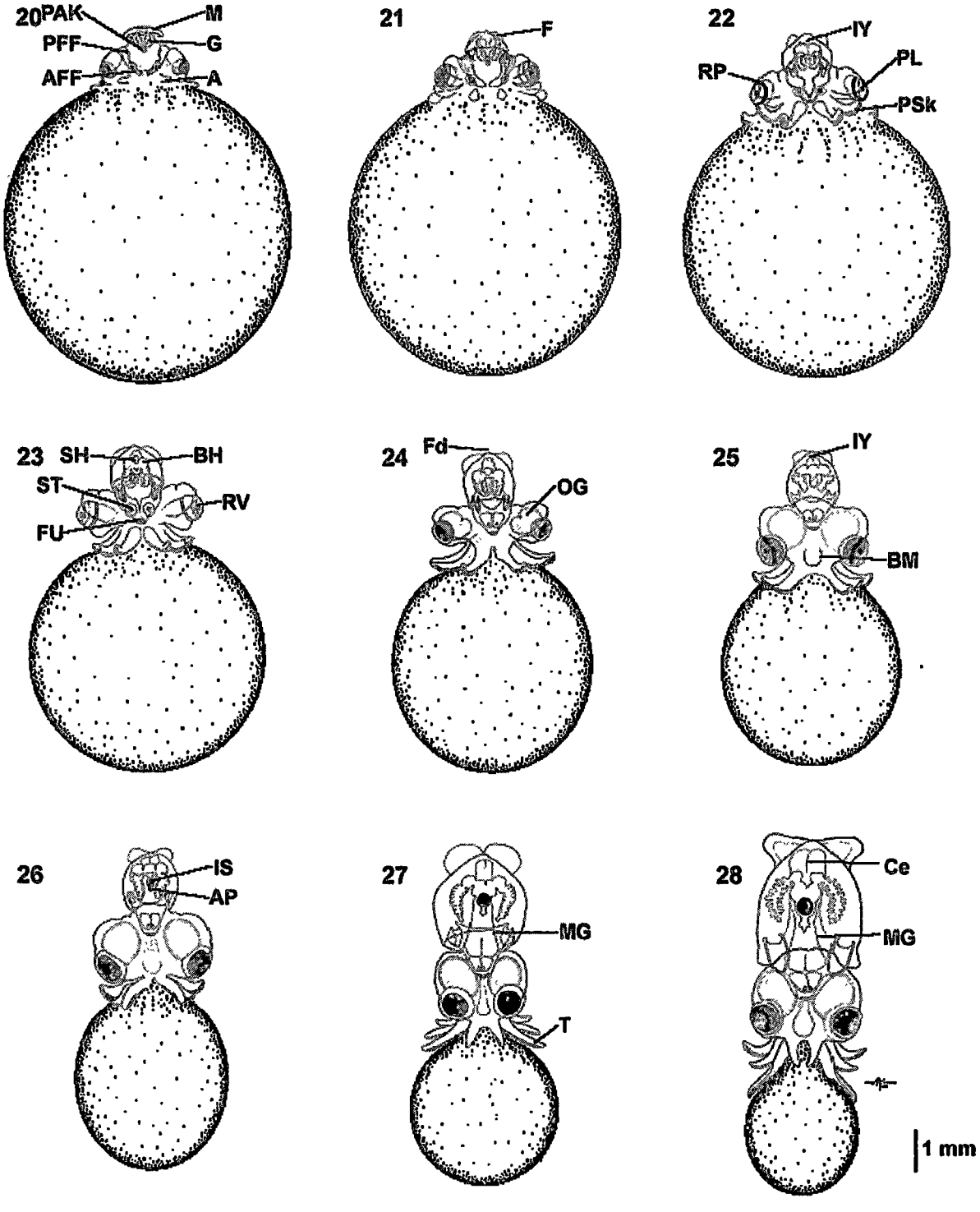


Figure 2.4. Illustrated developmental stages for *Sepioteuthis australis*. Stages 20-28 ventral views are represented. Refer to page 31 for abbreviation key.

Stage 29 (A29+, S29++)(Fig 2.5)

Green iridophores develop around the eyes. The olfactory organs are clearly evident on the head's ventral surface. E:Y ratio is 80:20. Embryos are increasingly active within the egg capsule and easily hatch leaving behind the reduced external yolk mass. Preserved pre-maturely hatched specimens display distinct countershading where the dorsal surfaces are more densely covered with dark chromatophores compared with ventral surfaces.

Stage 30 (A30; S30)(Fig 2.5)

Embryos have hatched having absorbed all or the majority of their external yolk stores. Hoyle's organ begins to deplete. After hatching they typically swim to the surface and are capable of inking, cryptic colour changes and behavioural postures, including V postures and upward curls as described in Moynihan and Rodaniche (1982) for *S. sepioidea* hatchlings

2.4.2. Species comparisons

The chronological appearance of organs in *Sepioteuthis australis* was similar to the basic loliginid scheme with only subtle heterochronies (Fig. 2.6). In general, the embryological process most closely resembled that of its tropical congener *S. lessoniana*, with the majority of heterochronies varying by only one embryological stage. For example, the opening and closing of the eye vesicle occurred one stage later, whereas the appearance of ventral chromatophores, anal papillae, and a full ink sac developed one stage earlier. The most notable difference between these two species was the appearance of Hoyle's organ, which appeared two stages earlier in *S. australis*. When comparing *S. australis* to *L. forbesi*, developmental differences were slightly greater. In this case, the appearance of the majority of structures were delayed by a few stages, with the exception of the shell gland, statocyst, lens primordia and closure of the shell gland which occurred synchronously and the appearance of the anal papillae and full ink sac which occurred one stage earlier. *Sepioteuthis australis* development however, was most different to the developmental scheme of *Loligo pealei*, where the appearance of certain structures was separated by more than three embryological stages (Fig 2.6).

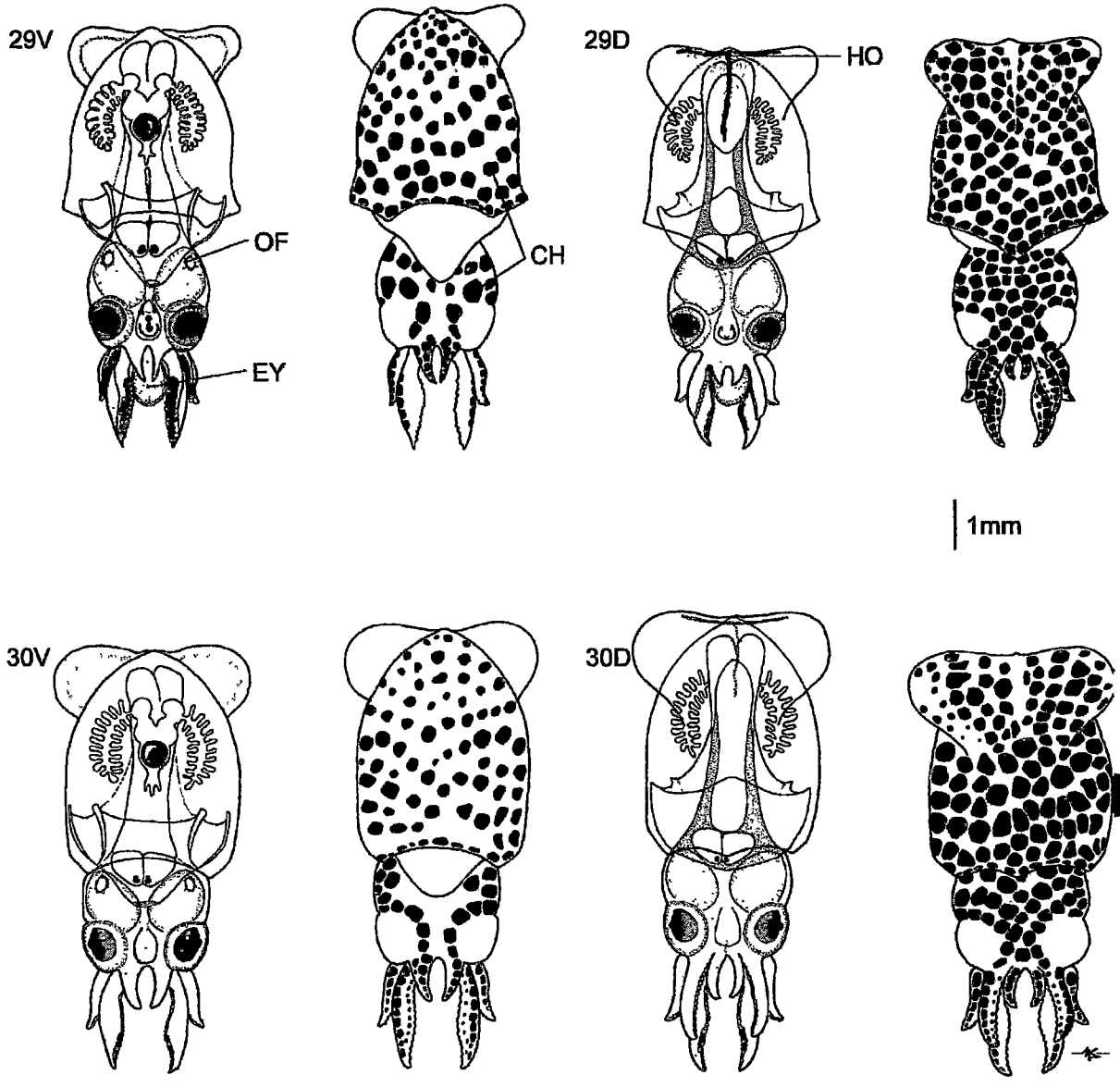
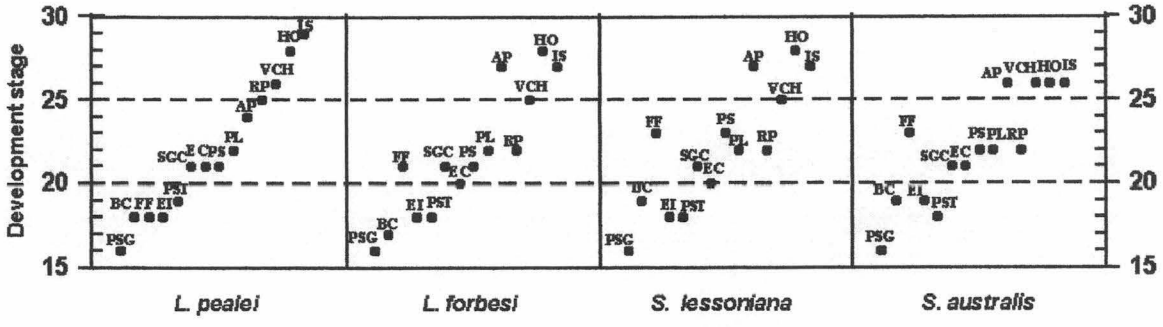


Figure 2.5. Illustrated developmental stages for *Sepioteuthis australis*. Stages 29-30, pre-mature and mature hatchlings. Both dorsal (D) and ventral (V) views are represented accompanied with illustrations of the chromatophore patterning determined from preserved (70% ethanol) specimens. Refer to page 31 for abbreviation key



Kendall's	*L. pealei*	*L. forbesi*	*S. lessoniana*	*S. australis*
L. pealei	1			
L. forbesi	0.824**	1		
S. lessoniana	0.686**	0.849**	1	
S. australis	0.703**	0.849**	0.935**	1

Figure 2.6. Comparison of the chronological appearance of organs in four Loliginids. Including a Kendall's tau correlation matrix. ** denotes significance at the 0.01 level (2-tailed). See page 31 for abbreviation key.

2.4.3. Effect of egg mass size and capsule position on embryonic development

There were strong position effects on the rates of embryonic development as a function of both the position of the egg strand within an egg mass and within an individual strand ($F_{mass*strand} = 2.517$, df = 5, 225, P = 0.031). Embryos developing at the proximal end of the strand displayed consistently slower developmental rates compared with their distal siblings (Fig 2.7). This trend was exaggerated for embryos developing within the internal strands as they displayed significantly higher variation, with proximal embryos on average developing 1.5 developmental stages behind distal embryos, compared to those in peripheral strands where only a 0.6 developmental stage difference was evident. Internal strands also displayed distinct signs of deterioration and decay compromising the integrity of the entire strand, whereas the peripheral strands were relatively unaffected.

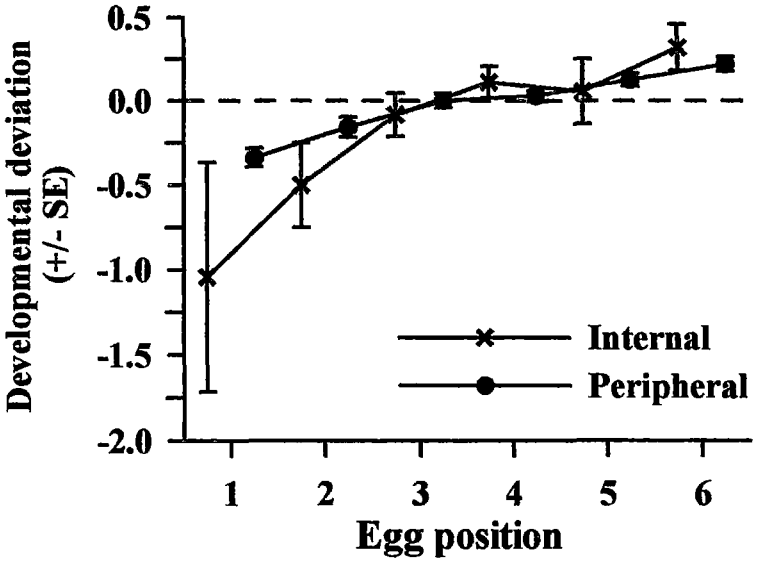


Figure 2.7. Within strand developmental deviation comparing internally and peripherally located egg strands. Egg position 1 refers to the proximal embryo and position 6 the distal embryo. Dashed line represents synchronous development within an egg strand, error bars represent standard error.

Internally located embryos were observed to develop significantly slower than peripheral embryos regardless of egg mass density (*cf.* average developmental stages 23.96 ± 0.05 with 24.60 ± 0.03; $F = 54.74$, $df = 1, 476$, $P < 0.001$). Significant differences in developmental rates were evident across masses of varying densities ($F = 11.57$, $df = 4, 476$, $P < 0.001$) with the smaller, less dense, egg masses harbouring embryos that were developing significantly faster than larger masses (Fig 2.8). This translates to a 1.5 developmental stage difference between the smallest (five strand) and largest (200+ strand) egg mass. Developmental rates did not, however, decrease linearly with increasing mass density as embryos collected from masses comprised of 200 strands were developing at a similar rate to those within the 50 strand mass (*cf.* average developmental stages 24.46 with 24.50).

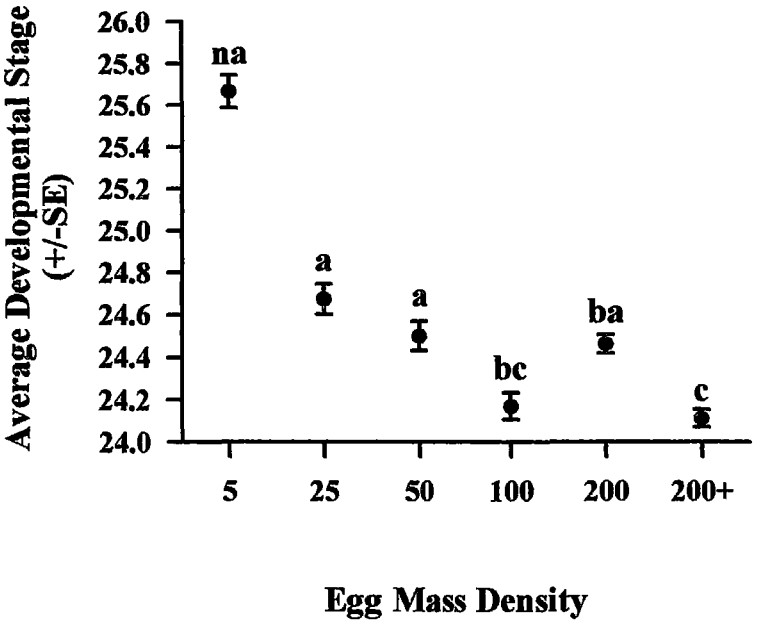


Figure 2.8. Developmental differences across egg masses of varying density, incorporating the Hochberg GT2 post hoc test (lower case letters). Means sharing the same letter are not significantly different. na denotes treatment not included in statistical analysis.

2.5. DISCUSSION

Rates of embryonic development determined for *S. australis* in this study are considered conservative estimates as they were calculated from the time of collection (Stage 12) rather than from the time of fertilisation. Nevertheless there were considerable differences in developmental rates for embryos reared at different temperatures, taking 52 days at 16°C and a further 9 days at 13°C. An inverse relationship between duration of development and incubation temperature is evident in the majority of cephalopod species, providing that temperatures do not fall outside the species' thermal tolerance boundaries (Sakurai et al. 1996; Oosthuizen et al. 2002). However, there is considerable variation between species, which is attributed to the respective size of the ovulated eggs, with species laying larger eggs displaying longer developmental times compared to species laying smaller eggs and incubated at similar temperatures. Of the loliginids, *Sepioteuthis* spp. produce the largest eggs (eg *S. australis* 5.6-7.5 mm (Pecl 2001); *S. lessoniana* 5.6-5.8 mm (Segawa 1987)) compared to *Loligo* spp. (eg *L. forbesi* 3.0-3.3 mm (Segawa et al. 1988)); *L. pealei*

1.0-1.6 mm (McMahon and Summers. 1971)). It is therefore possible that ovulated egg size and subsequent developmental rates may be responsible for the observed heterochronies. This is consistent with Baeg et al. (1992), however there is some conjecture in defining differences as true species specific heterechronies as they may be an artefact of differing observation techniques or rearing conditions (Guerra et al. 2001).

Increasing egg mass density affected variation in the rate of embryonic development within egg strands for laboratory reared calamary eggs. This was particularly evident for those embryos located within the centre of the aggregated mass as they displayed greater developmental deviation than those strands located around the periphery. This trend may be directly attributed to diffusion problems resulting from the negative effect of crowding embryos. Typically embryos located either deep within aggregated egg masses or close to the substrate where there is little to no surface exposure, experience problems acquiring oxygen and eliminating metabolic wastes as demonstrated in the pond snail *Lymnaea stagnalis* (Marois and Croll 1991). Pechenik (1986) suggests that these diffusion problems may play a role in limiting egg mass morphology, size and number of capsules packaged within each strand. The susceptibility of these embryos will therefore be directly proportional to the density, or crowding of the egg mass. Although no significant interaction between egg mass density and strand locality was evident in this study, our results consistently displayed different developmental rates within each egg mass regardless of density, where internally located strands developed approximately 1.0 developmental stage behind peripheral strands.

It is possible that the results obtained in this study are inflated due to embryos being incubated in controlled, artificial laboratory conditions not indicative of the natural environment. Obviously the developmental process hinges on a variety of other environmental factors and estimates would theoretically be reduced if tidal currents and wave action flushed egg masses. There is field evidence, however, of asynchronous development and high incidences of dead embryos located close to the egg mass' attachment point in *S. australis* (Steer et al. 2002; Chapter three). Furthermore, there have been instances where central strands within large egg masses have displayed signs of deterioration and decay (Steer, unpublished data).

These results raise interesting questions relating to the spawning behaviour of female loliginids. For example, what are the benefits (in terms of embryonic success) of multiple females contributing to an existing egg mass and therefore increasing its relative density? Multiple females do contribute egg strands to a common egg mass for *L. pealei* (Arnold 1962), *L. opalescens* (Hixon 1983), *L. v reynaudii* (Sauer et al. 1992) and *S. australis* (Jantzen and Havenhand 2002). However, it is not clear where on the existing egg mass the new strands are attached. Adding egg strands to the periphery of existing masses would theoretically maximise embryonic development and hatching success as the female effectively avoids centrally located strands. Laying small discrete masses consisting of long egg strands (i.e comprised of many eggs) would similarly be beneficial. Laying longer egg strands first will potentially buffer the effects of other females contributing to the mass, as the embryos located at the distal ends of the strand are least susceptible to the constraints associated with an aggregated mass.

2.6. ABBREVIATION KEY

A	Arm	**Mth**	Mouth
AFF	Anterior funnel fold	**OF**	Olfactory pore
AP	Anal papillae	**OG**	Optic ganglia
BC	Blastoderm completely cellulated	**OV**	Optic vesicle
BH	Branchial hearts	**PA**	Arm primordia
Bl	Blastoderm	**PAK**	Anal knot primordium
BM	Buccal mass	**PFF**	Posterior funnel fold
Ce	Caecum	**PG**	Gill primordia
CH	Chromatophores	**PL**	Lens primordia
Cn	Chorion	**PM**	Mantle primordium
EC	Eye vesicle closes	**PSG**	Shell gland primordium
EI	Eye vesicle invaginates	**PS**	Sucker primordia
EY	External yolksac	**PST**	Statolith primordia
F	Fins	**RP**	Retinal Pigmentation
Fd	Fused Fins	**RV**	Retinal vesicle
FF	Funnel Fuses	**SG**	Shell gland
FU	Funnel	**SGC**	Shell gland closes
G	Gills	**SH**	Systemic Heart
HO	Hoyle's organ	**ST**	Statolith
IS	Ink sac	**T**	Tentacle
IY	Internal yolksac	**VCH**	Ventral chromatophores
M	Mantle	**Y**	Yolk
MG	Midgut gland		

CHAPTER THREE

TEMPORAL VARIABILITY IN EMBRYONIC DEVELOPMENT AND MORTALITY IN THE SOUTHERN CALAMARY *SEPIOTEUTHIS AUSTRALIS*: A FIELD ASSESSMENT

Steer, MA., NA Moltschaniwskyj, FC Gowland. (2002). Marine Ecology Progress Series 243: 143-150

3.1. ABSTRACT

This study describes the incidence of embryo mortality and differential development in southern calamary (*Sepioteuthis australis*) eggs. Late stage *S. australis* egg strands containing multiple embryos close to hatching were sampled from shallow (<4m) Tasmanian spawning grounds from early November 2000 to January 2001. Results indicated that *S. australis* embryos develop asynchronously within individual egg strands with proximal embryos developing slower and suffering higher mortality than their distal siblings. The magnitude of asynchrony however differed throughout the season with greater within-strand differences observed when embryos were exposed to broader environmental temperatures. Unexpectedly embryos developed more synchronously within biologically fouled strands and displayed a significantly lower incidence of mortality compared to those developing in unfouled strands. Embryo mortality was initially low (4%) and significantly increased to 20% in late November remaining above 10% until late December. This dramatic increase in mortality was not strongly associated with increasing water temperatures but coincided with a period of heavy rainfall followed by relatively calm inshore conditions.

3.2. INTRODUCTION

Quantitative estimates of mortality rates for squid species are virtually non-existent. However they have been vaguely defined as higher than mammals, similar to marine plankton and lower than fish (O'Dor 1998). This description arises purely as a function of the logistical and technical complexities associated with reliably sampling the animals through the egg, paralarval and juvenile phases in the wild. However, the need to quantify early mortality rates and identify processes responsible is important, especially from a fisheries management perspective, as it can potentially be used to predict recruitment strength.

For most fish species egg and larval mortality rates are generally high, but extremely variable (Ferron and Legget 1994). This variability is largely attributed to

the larva's vulnerability to physical and biological interactions, e.g., predation pressure, starvation and the physical environment (Sissenwine 1984). Such variability contributes significantly to variation in recruitment to the adult population (Narimatsu and Munehara 1999). Loliginid squid, however, differ fundamentally from fish, as they do not exhibit a true larval phase (Young and Harman 1988). Instead they undergo direct embryonic development within well-protected, sedentary egg capsules to hatch as behaviourally and structurally adept hatchlings (Boletzky 1987, Boyle et al. 2001). As a consequence some of the risks associated with a planktonic metamorphosis phase are reduced resulting in higher survivorship than many marine fishes (Caddy 1983).

Unfortunately direct embryonic development in squid is typically a lengthy process, representing up to 30% of their short lifespan, depending on the species (Boletzky 1987). During this time the developing embryos are potentially at risk to fluctuating environmental conditions (Augustyn et al. 1994). Temperature defines embryonic developmental limits (Segawa 1988) and influence rates of development (Boletzky 1994) and is therefore considered the principal environmental factor governing cephalopod embryonic development. Although some loliginid squids spawn sporadically during the winter months or in deep, cold water (e.g. *Loligo forbesi* Lordon and Casey 1999, *Loligo. gahi* Arkhipkin et al. 2000) most species aggregate with some predictability in shallow waters during warmer months to spawn (Hanlon 1998). Spawning during this time and in these regions is assumed to maximize hatching success and survival by effectively accelerating developing embryos through the early vulnerable phase. However, the developing embryos are still potentially vulnerable to rapid temperature and salinity fluctuations resulting from prevailing weather conditions, whilst also being at risk of dislodgment due to storm activity (Augustyn et al. 1994, Moltschaniwskyj and Pecl 2003) and excessive biofouling.

In the laboratory, large fluctuations in temperature and salinity are responsible for major structural deformities and high embryo mortality (Boletzky and Hanlon 1983, Palmegiano and D'Apote 1983; Hanlon 1990; Ueta et al. 1999). In the wild, survivorship may be affected by epiphytic growth on the egg strands' surface, especially for those laid in shallow, nutrient-rich waters and during the later stages of

development (Moltschaniwskyj et al. 2002). The effect of epiphytic growth on the developing embryos is unclear, but it may contribute to 'unsatisfactory hatching' (Choe 1966). To date, the relative importance of biotic and abiotic factors on egg development and survival in nature has not been directly assessed due to the difficulties associated with *in situ* investigations. The southern calamary, *Sepioteuthis australis*, however, reliably aggregates in shallow protected waters on the east coast of Tasmania to mate and spawn, allowing researchers regular access to extensive spawning beds. Spawning in *S. australis* is typical of the loliginids; once mated, females attach a series of digitate egg strands to seagrass/macroalgae holdfasts to collectively form discrete egg masses. Individual egg masses may comprise <10 to >500 strands, with each strand containing between 2 and 8 longitudinally aligned egg capsules (Moltschaniwskyj and Pecl 2003).

This field-based study aimed to quantify variability in rates of development and mortality for calamary embryos throughout a spring/summer-spawning season. Due to the collective egg packaging strategy exhibited by this species, this study also described variation in development within individual egg strands to determine if certain eggs were at a higher risk to mortality. Furthermore, the effect of biofouling on the capsule surface and its effect on mortality rates were quantified.

3.3. Materials and Methods

Four southern calamary spawning sites located on the east coast of Tasmania, Australia (42°07'34"S, 148°17'51"E) were visited fortnightly from early November 2000 until early January 2001. All sites were within 5 km of each other, within 100 m of the shoreline, less than 4 m depth and subjected to a maximum tidal range of 1.2 m. On each trip divers searched areas of *Amphibolis antartica* seagrass beds for egg masses containing embryos close to hatching. From each egg mass three biofouled (F) egg strands (75-100% of the surface supporting filamentous algae) and three unfouled (UF) egg strands (0% of the surface supporting filamentous algae) were collected, bagged and stored in fresh seawater prior to dissection (Fig 3.1).

3.3.1. Dissections

Embryos were dissected from each strand within 8 hours of collection and examined under a stereo dissection microscope. Developmental stage was assigned to each embryo according to the criteria described by Steer et al. (2003) (Chapter two) that differs slightly (in terms of the chronological appearance of the eye vesicle, ventral chromatophores, anal papillae and ink sac) from the developmental scheme proposed by Segawa (1987) for *Sepioteuthis lessoniana*. The position of each embryo within an egg strand was recorded; position 1 identified the embryo located at the fixed/proximal end of the strand and progressing consecutively to the free/distal end of the strand. Eggs that were unfertilised, had ceased development, or were undergoing abnormal development were scored as "dead". Due to the external fertilisation process exhibited by spawning females the incidence of unfertilised eggs was expected to be negligible and therefore not confound mortality estimates. To avoid over-estimating the within strand developmental differences, all embryos that had hatched to leave an obviously vacant egg capsule were assumed to have hatched prematurely (stage 29). This assumption was based on the observation that premature hatching readily occurs during collection (pers obs).

Figure 3.1. Three unfouled and three fouled *S. australis* egg strands. Within strand egg position indicated.

3.3.2. Environmental data

Seawater temperature was measured within seagrass beds using 32K StowAway® TidbiT® temperature dataloggers located at three collection sites. Dataloggers were secured to the substrate (<4m deep) one month prior to the first sampling occasion and logged temperature hourly. Daily rainfall data (as a proxy of salinity) were obtained from a local weather station maintained by the Australian Bureau of Meteorology.

3.3.3. Back-calculated oviposited dates

To examine conditions experienced by developing embryos sampled on each trip we back-calculated the approximate date the eggs were laid. Oviposition dates were calculated using Laptikhovsky's (1999) predictive equation for decapods. This equation takes mean egg size (L mm) and incubation temperature (T °C) in consideration to generate the duration of embryogenesis (D days). The equation was applied as follows:

$$D = (1220.94 \cdot T^{-1.68194}) L^{k},$$
$$\text{where } k = 2.5139 \cdot T^{-0.3574}$$

Two problems were evident in this equation. Firstly, as the predictive estimates are calculated for eggs developing under constant temperature regimes, they do not account for unknown effects of natural temperature fluctuations observed in the field. Secondly the equation does not incorporate development stage at the time of collection, but rather calculates the time to hatching. In an attempt to counteract these problems T was derived from the average field temperature recorded 3-weeks prior to collection and mean egg development stage at time of collection was factored in to provide approximate oviposition dates (Table 3.1). Egg length (L) was determined by measuring a total of 375 ovulated eggs collected from the ovaries of 13 mature females (mean 6.18 ± 0.06 SE).

Table 3.1. Back-calculated oviposition date for field-collected *Sepioteuthis australis* eggs using Laptikhovsky's (1999) model. Mean incubation temperature three weeks prior to collection and developmental stages at time of collection are factored into the equation.

Date Collected	mean temp Prev 3 weeks (°C)	Predict dev time until hatch (days)	Average Dev Stage	Predict dev time At collection (days)	Approx. date laid
08-Nov-00	14.5 ± 0.04	78.8	27.3 ± 0.10	72.3	28-Aug-00
22-Nov-00	15.2 ± 0.05	70.7	26.5 ± 0.08	55.9	27-Sept-00
6-Dec-00	17.3 ± 0.04	52.9	26.2 ± 0.09	40.7	20-Oct-00
21-Dec-00	18.7 ± 0.05	44.2	27.8 ± 0.12	38.8	10-Nov-00
08-Jan-01	18.0 ± 0.07	48.2	27.9 ± 0.08	43.9	24-Nov-00

3.3.4. Statistical analysis

To determine variability in embryonic development within egg strands the average development stage and the deviation of each embryo from the strand average was calculated for all egg positions. If embryonic development was synchronous within an egg strand, each embryos' deviation from the strand mean would be zero. These differences in developmental deviation within strands were then examined as a function of sampling time, biofouling and position within the strand using a 3-factor Model 1 ANOVA. Only egg strands containing 4 and 5 eggs were included in the analyses to avoid severely unbalancing the dataset. All dead embryos were included in the analysis as missing values as developmental stage could not be ascertained. Assumptions of ANOVA were checked by visual inspection of variance and normality plots. No data transformations were required.

To determine if developmental asymmetry was a function of the number of eggs within a strand, the difference in developmental stage between the fixed and free ends was calculated for all strands containing between 4 and 7 eggs (n = 226). Strands containing the extremes (3 and 8 eggs) were not included in the analysis due

to insufficient replication. A one-way ANOVA with unequal replication was used to compare mean values. The Hochberg GT2 post hoc test for an unbalanced dataset (Sokal and Rohlf 2000) was used to highlight significant differences amongst means.

Temporal effects on the frequency of mortality as a function of biofouling and the eggs' respective position within the egg strand were estimated via a series of non-parametric log-likelihood ratio (G) tests. Incorporation of Williams's correction (G_{adj}) ensured a more conservative estimate of G and therefore reduced the risk of type I errors (Sokal and Rohlf 2000).

3.4. RESULTS

Rates of development of *Sepioteuthis australis* embryos within a strand differed among egg positions, but the pattern and magnitude of the difference along the strand depended upon time ($F = 3.48$, $df = 16, 601$, $P < 0.001$). On most occasions the embryos located at the fixed (proximal) end of the strand developed more slowly than those located at the free (distal) end (Fig 3.2). However, in late November position 2 embryos were slowest to develop, on average lagging 0.7 developmental stages from the distal embryos (Fig. 3.2). The within-strand difference in development was greatest for those eggs collected in early December with proximal embryos on average lagging 1.1 developmental stages behind distal embryos. This difference coincided with the largest incremental change in water temperature, where embryos were subjected to a 7.3°C increase over a 41-day developmental period (Fig. 3.3a). Development rates of embryos within the strand were most synchronous in early November where proximal and distal embryos exhibited a mean difference of 0.4 in developmental stage (Fig 3.2). Temperatures experienced by developing embryos collected in early November were relatively constant with embryos experiencing a 3.8°C range throughout development (Fig 3.3a). There was a weak positive correlation between within-strand development variation and rate of temperature change (Pearson's correlation, $r = 0.87$, $n = 5$, $P = 0.05$).

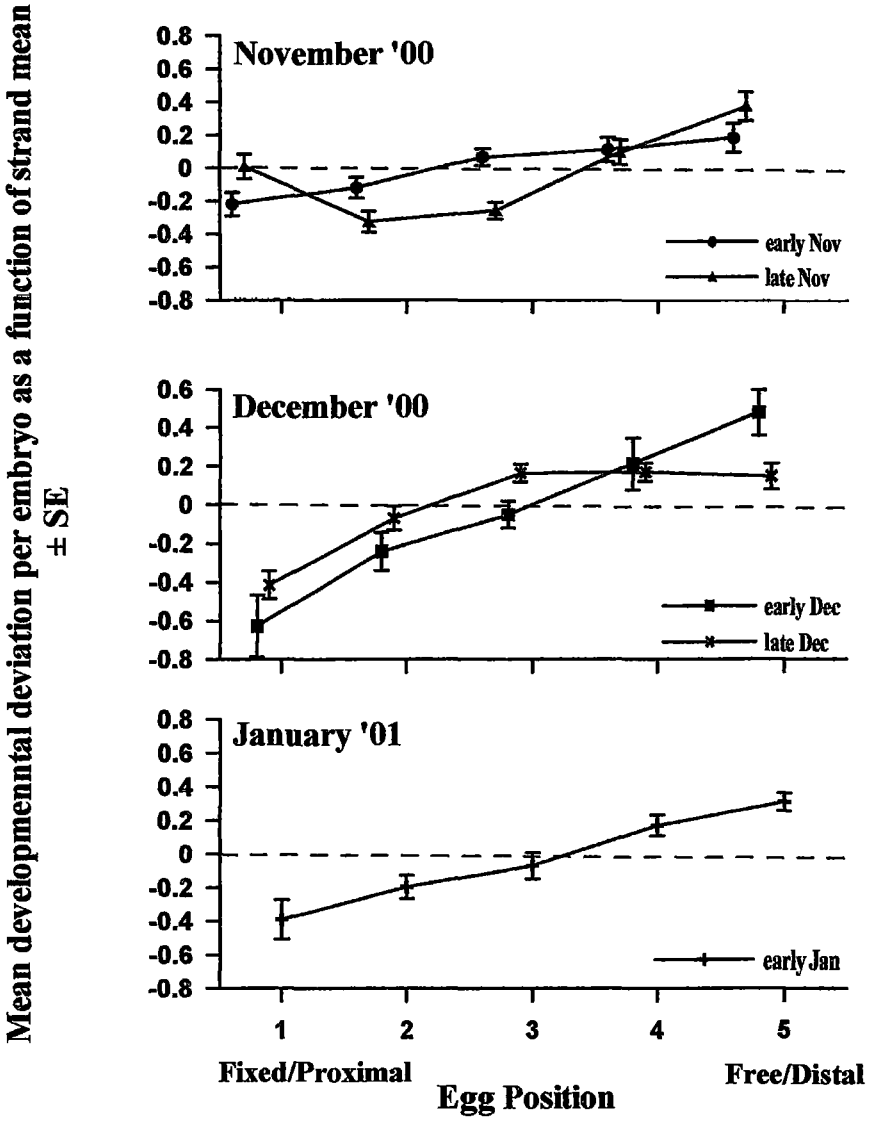


Figure 3.2. Mean differences in the development of *Sepioteuthis australis* eggs during a spring/summer spawning season. Mean developmental deviation per embryo is calculated as a function of strand mean. Only egg strands containing 4 or 5 egg capsules were included in the analysis (n = 226). Dashed line represents synchronous development within an egg strand, error bars represent standard error.

Fouling on the egg strands had a large effect on rates of development within the strand, with greater within-strand variation in unfouled than in fouled egg strands ($F = 13.76$, $df = 4, 601$, $P < 0.001$). While proximal embryos in unfouled egg strands on average lagged 1.0 development stage behind distal embryos, those in fouled embryos lagged by only 0.4 (Fig. 3.4).

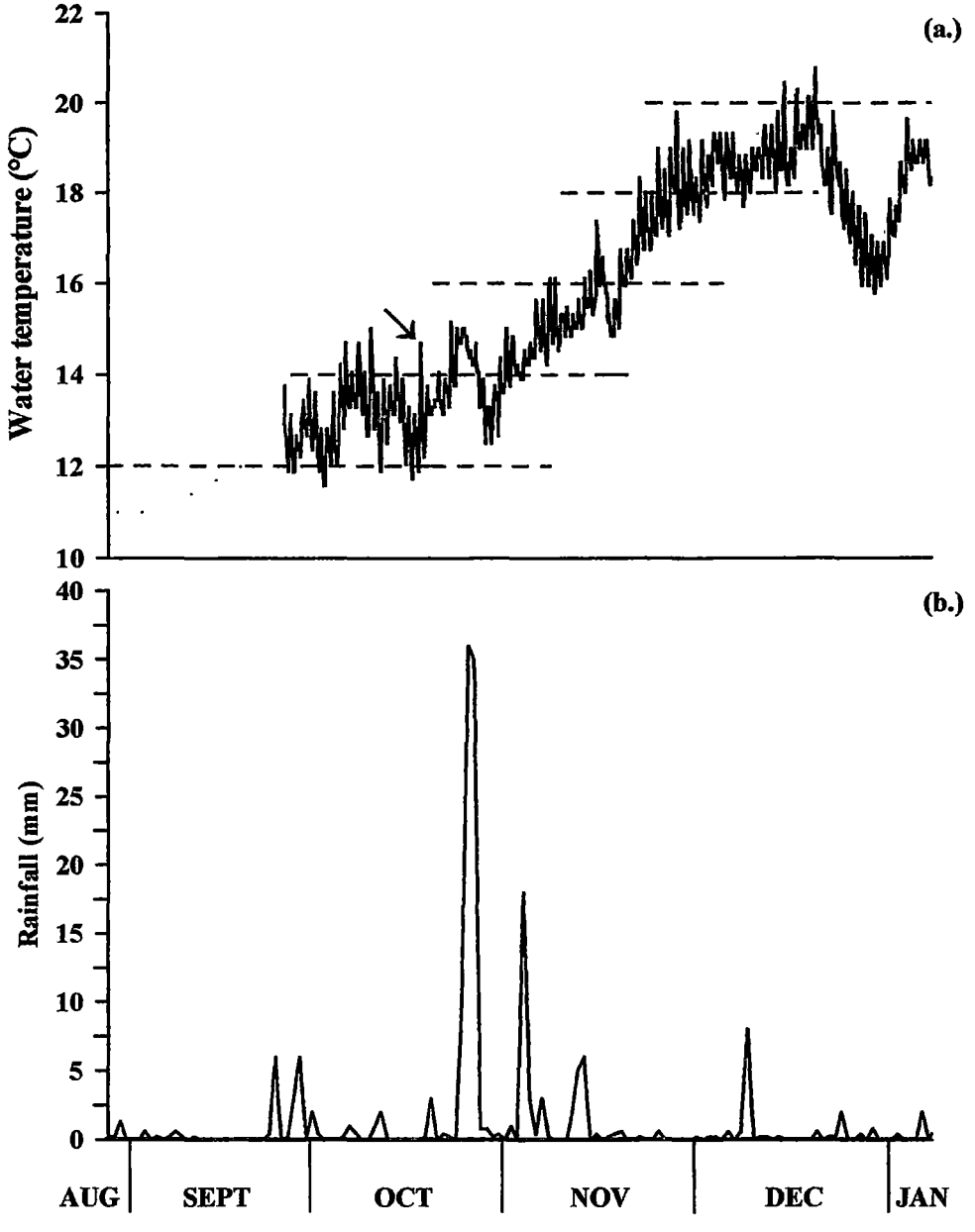


Figure 3.3. (a.) Benthic water temperature (°C) measured hourly; dashed horizontal lines represent duration of embryonic development of collected egg samples calculated using Laptikhovsky's (1999) predictive equation for decapods. Arrow indicates the greatest temperature spike observed during the study period. (b.) Daily rainfall (mm).

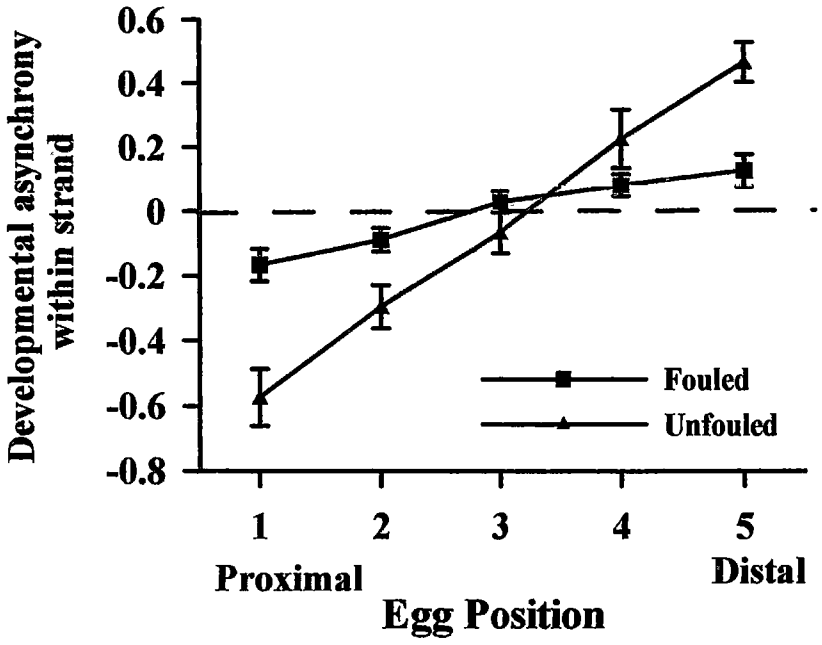


Figure 3.4. Mean differences in the development of *Sepioteuthis australis* eggs between fouled and unfouled strands. Mean developmental deviation per embryo is calculated as a function of strand mean. Where fouled = 75-100% surface coverage, Unfouled = 0% surface coverage. Dashed line represents synchronous development within an egg strand, error bars represent standard error.

The number of eggs in the strand affected the difference in the rate of development between the proximal and distal end ($F = 3.31$, $df = 3, 222$, $P = 0.021$). The shortest egg strands showed minimal difference, with proximal embryos lagging 0.57 ± 0.17 developmental stages behind distal embryos. Longer egg strands, however were observed to display greater asynchrony in development with 1.2 ± 0.2 developmental stages separating proximal and distal embryos.

Incidence of embryo mortality changed over the summer, ranging from ~4% dead in early November to 19.5% in late November (Fig 3.5). Total mortality remained above 10.0% in early December and January periodically dropping to 6.4% in late December, however greatly varied as a function of biofouling ($G_{adj} = 89.27$, $df = 3$; $P < 0.001$). From late November onwards the incidence of mortality in unfouled egg

strands was as much as 90% higher than that in fouled strands. Except in early November mortality rates were very similar in both fouled and unfouled egg strands.

Using Laptikhovsky's (1999) predictive equation it was possible to back-calculate the approximate oviposited dates from sampled embryos. Embryos collected in the initial sample were back-calculated to be laid on the 28th August marking the 'beginning' of the study period (Table 3.1). By calculating oviposited date it is possible to identify potential environmental perturbations throughout embryonic development (ie from when eggs were laid to when they were sampled) (Fig 3.3a). Diel temperature fluctuations were small throughout the study (mean 1.18°C ± 0.55 SD). The largest temperature change occurred on 17th October where there was an increase of 2.8°C over a 7-hour period (Fig 3.2a). All embryos collected in November would have experienced this subtle temperature spike during their developmental process, however, there was no correlation with increased mortality rates and changes in water temperature throughout the study (Pearson's correlation, $r = 0.19$, $n = 5$, $P = 0.49$) (Fig 3.3a). Embryos collected in November and early December experienced a period of heavy rainfall where a total of 83.2 mm fell over 7 days (from 24th to 30th October, with major downpours occurring on the 25th and 26th measuring 36 and 35 mm respectively (Fig 3.3b). Rainfall during this period considerably exceeds the October average of 50 mm (Australian Bureau of Meteorology).

Embryos developing at the proximal end of an egg strand suffered mortality rates more than seven times higher than those at the distal end, a pattern that was consistent in both fouled and unfouled strands ($G_{adj} = 147.74$, $df = 7$, $P < 0.001$) (Fig 3.6). No dead embryos were found in positions 7 or 8 regardless of biofouling (Fig 3.6).

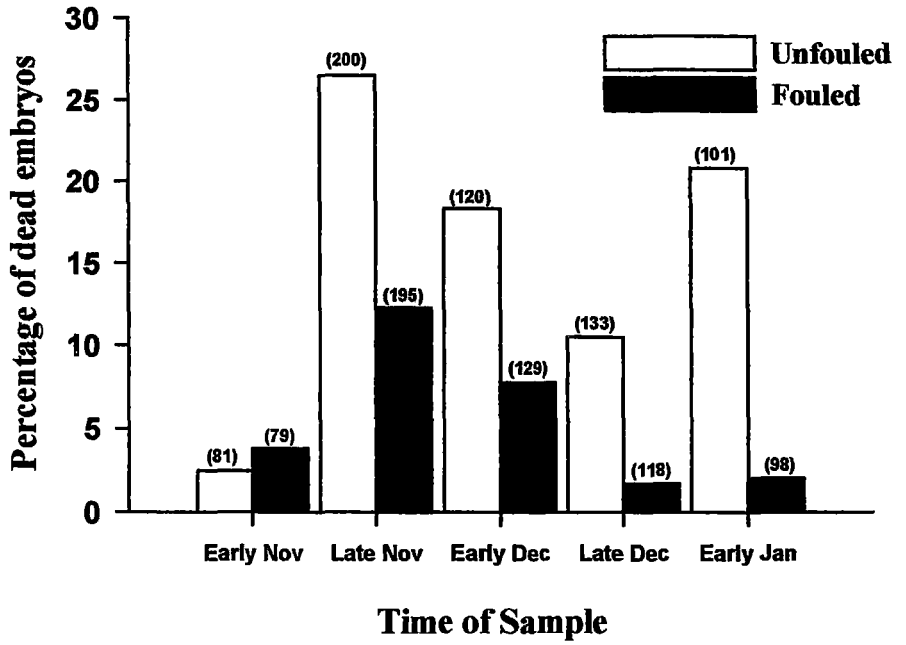


Figure 3.5. The percent frequency of dead embryos at each sampling time across all strand positions for fouled and unfouled egg strands. Numbers in parentheses indicate total number of embryos examined.

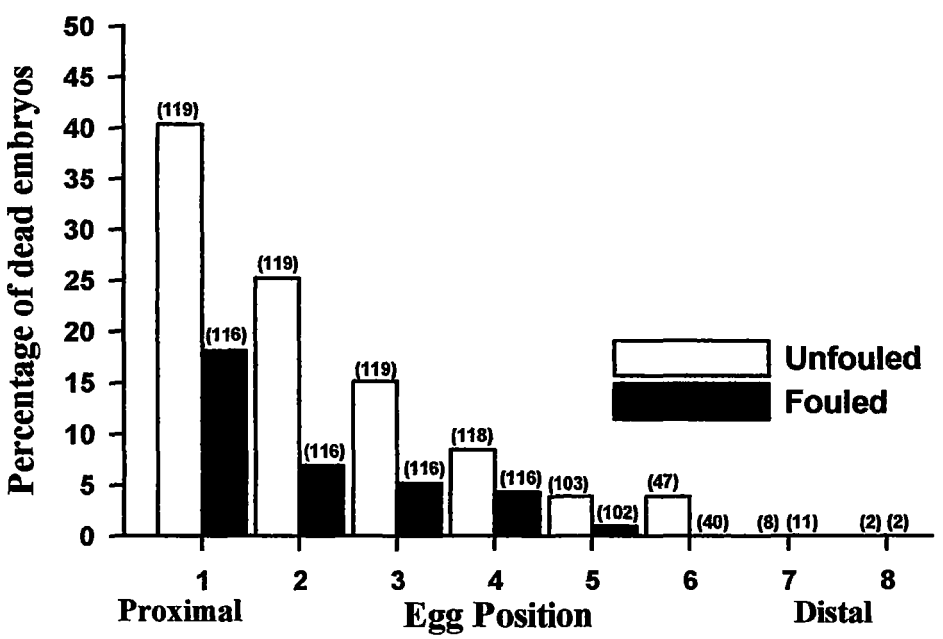


Figure 3.6. The percent frequency of dead embryos at each position along the egg strand for fouled and unfouled egg strands. Numbers in parentheses indicate total number of embryos examined.

3.5. DISCUSSION

Southern calamary embryos develop asynchronously within individual egg strands with proximal embryos consistently developing slower and suffering higher mortality than their distal siblings. Variable development within an aggregated egg mass has been reported in a variety of marine species e.g. gastropods *Lymnaea stagnalis, Melanochlamys diomedeai* and *Haminaea vesicula*, polychaete *Nereis vexillosa* (Marois and Croll 1991, Cohen and Strathmann 1996), and fish *Clupea harengus* (Stratoudakis et al. 1998). In each instance embryos in the distal or outer perimeter of the egg mass develop faster and hatch first. This difference in developmental rate is attributed to the interior embryos not getting sufficiently oxygenated and/or accumulating excess excretory products and responding by either retarding or arresting development (Strathmann and Strathmann 1995). Given the high density of eggs in the squid egg masses, particularly at the attachment point, it would be of value to determine the micro-environmental characteristics experienced by the embryos.

Temporal variations in developmental rate and mortality estimates were explored as a function of incubation temperature. The positive correlation between the magnitude of within strand developmental variation and incubation temperature range suggests that asynchronous development was greater when embryos were subjected to a wider temperature range. Importantly, this can translate to differential hatch times, suggesting that an entire egg mass will hatch over a longer period when incubation temperatures change, compared to egg masses experiencing relatively stable temperatures. Similar results have been observed in shallow-water, benthic spawning fish (e.g. Pacific Herring *Clupea pallasii* and Capelin *Mallotus villosus*), where the hatching interval was negatively correlated with the average incubation temperature (Taylor 1971; Frank and Leggett 1981). Given that temperature intrinsically defines developmental rates it is likely that when embryos are subjected to progressively warmer temperatures, typical of a seasonal increase, embryonic development speeds up, exaggerating the within strand variation. It is unclear if survival of remaining embryos is compromised once hatching begins.

Although seasonal increases in temperature are positively correlated with developmental rate their influence on embryo mortality remains unclear. Temporal differences in embryo mortality have been observed in the long-finned squid *Loligo gahi* where the incidence of mortality during the winter months is more than four times greater than during spring and summer (Arkhipkin et al. 2000). It remains to be investigated whether this seasonal difference was purely a result of differing temperatures, or other environmental conditions, or a combination of both. Extreme temperature fluctuations are suggested to be detrimental in laboratory reared eggs, especially during the earlier developmental stages, and controlled temperature shifts are advised not to exceed 1°C per day (Hanlon 1990). As temperature fluctuations during this study were generally between 1 and 2°C per day it is unlikely that thermal conditions contributed to elevated mortality. A previous study of late stage *Sepioteuthis lessoniana* embryos showed that short-term exposure (<1 hr) to temperatures 3-7°C above natural conditions does not adversely affect development (Kinoshita 1982). As the maximum rate of temperature elevation recorded in this study equated to 2.8°C over a 7-hour period, it is unlikely to have promoted developmental arrest. A study by Pedersen and Tande (1992) suggest that invertebrates living in environments characterised by natural increases in temperature during the developmental period are physiologically adapted to cope with slight fluctuations. By this rationale *Sepioteuthis australis* embryos may be relatively robust with regard to coping with temperature fluctuations.

Heavy rainfall and subsequent fresh-water run-off, in October may have contributed to elevated mortalities observed in late November and early December. As all eggs examined in this study were collected in shallow, nearshore waters (< 4m) their development may have been perturbed by rapid changes in salinity. Cephalopods are generally thought to be stenohaline and supporting field evidence suggest that low salinities have a significant inhibitory effect on hatching success in the cuttlefish *Sepia officinalis* (Palmegiano and D' Apote 1983). Embryos that were collected in early Novemeber, and would have experienced this change in salinity, however, displayed a relatively low incidence of embryo mortality, suggesting that if salinity was to have an effect on development, the timing of such events may prove to be an important factor.

Biofouling did not seem to have any obvious detrimental effect upon embryonic development. Embryos developing within fouled strands displayed relative synchrony in development and a low incidence of mortality compared to those developing within unfouled strands. This result was unexpected, as epiphytic growth upon amphibian eggs typically creates hypoxic conditions during the night (Pinder and Friet 1994). It has been suggested that colonisation of the fouling organisms on *S. australis* egg strands is delayed by a chemical defense present on the strands' surface. Therefore any resultant growth is not rapid enough to interfere with development as embryos hatch in sufficient time before fouling has any affect (Benkendorff 1999). Conversely, the photosynthetic abilities of fouling organisms coupled with the surrounding *Amphibolis* vegetation may enhance oxygen levels during the day and water movement during the night may cancel out any negative effect of epiphyte respiration (Cohen and Strathmann 1996). Excessive biofouling may additionally benefit developing embryos by protecting them from potentially damaging solar radiation that has been known to cause problems in development in other taxa (Biermann et al. 1992) and perhaps explain why mortality was higher in strands that were free of fouling.

The present study demonstrates temporal variability in development rates of *Sepioteuthis australis* embryos. The dynamic nature of shallow water spawning sites makes it difficult to single out the major contributing factor(s) responsible for embryo mortality. As a result, future work should focus upon defining tolerance levels in developing embryos exposed to a variety of fluctuating environmental conditions and determine their relative effect on different phases of development. In addition, the size and density of an egg mass needs be factored into any analysis to determine if this is contributing to early mortality rates (see Strathmann and Strathmann 1995).

Commercial catches of *Sepioteuthis australis* off Tasmania have increased in recent years and a series of fishery closures aimed at protecting the spawning stock and maximizing recruitment strength were implemented (Moltschaniwskyj et al. 2002). Understanding the effect of the environment on embryonic development and hatching success can reduce some of the variability encompassed within existing

stock-recruitment relationships, which are currently based on spawner biomass/parental stock sizes. Reducing variability within predictive stock-recruitment relationships will allow fisheries managers to make more informed and accurate decisions about the fishery.

CHAPTER FOUR

FACTORS RESPONSIBLE FOR EMBRYO MORTALITY IN THE SOUTHERN CALAMARY *SEPIOTEUTHIS AUSTRALIS*: THE ROLE OF THE AGGREGATED EGG MASS

Steer, MA., NA Moltschaniwskyj, submitted Marine Biology

4.1. ABSTRACT

Using a combination of laboratory and field investigations this study examined the role of egg mass size, the substrate upon which the mass is attached, the position of the embryo within the mass and the degree of biofouling on embryo mortality in the southern calamary *Sepioteuthis australis*. Egg mass size ranged from 2 to 1241 egg strands, however most masses consisted of 200-299 strands. Small egg masses (<300 strands) were generally attached to soft-sediment vegetation (*Amphibolis antarctica*, *Heterozostera tasmanica*, *Caulerpa* sp.), whereas larger masses (>300 strands) were either securely attached to robust macroalgae holdfasts (*Ecklonia* sp., *Marcocystis pyrifera*, *Sargassum* sp.) or unattached. Rates of embryo mortality were highly variable, throughout the course of the study, ranging from 2 to 25%. Both laboratory and field results indicated a positive relationship between egg mass size and embryo mortality. Larger, unattached egg masses contained twice the amount of dead embryos than those securely attached to a substrate. Mortality rates were significantly affected by the embryos' relative position within the mass. Embryos located around the attachment point of the egg strand, within the interior of the mass, or in close contact with the substrate were more likely to die. This was attributed to the inability of the embryos to adequately respire and eliminated metabolic wastes within the constraints of the egg mass. Biofouling did not strongly influence embryo mortality, but colonised areas conducive to growth, photosynthesis, and respiration indicating 'healthy' regions within the mass.

4.2. INTRODUCTION

Cephalopod embryos developing in benthic eggs can be considered sedentary as they are effectively anchored throughout the course of development until they individually hatch. As a result developmental and mortality rates will largely be influenced by the immediate physical and biological environment. Site selection by spawning females can therefore contribute to the success of embryonic development and potentially the strength of the subsequent generation (O'Dor 1998). There are few field studies that have explored processes determining embryo mortality rates,

largely because of the logistical difficulties of sampling late stage embryos from egg masses *in situ.*

Most neritic squid (e.g. loliginid species) form large spawning aggregations and deposit clusters of eggs attached to a variety of substrates including differing vegetation types, coral, sand, and inanimate structures (Sauer et al. 1993; Segawa et al. 1993; Ueta and Kitakado 1996; Arkhipkin, et al. 2000; Moltschaniwskyj et al. 2002). Although intermittent spawning may occur throughout the year and in deeper water, spawning by loliginids typically peaks during warmer months and in shallow, protected embayments (Hanlon 1998). Consequently, it is assumed that these areas are preferred 'sanctuaries' for the embryos as they have sufficient substrate for egg attachment, are well aerated by wave activity, and display seasonal temperatures optimal for correct embryonic development (Augustyn 1990). This generalisation, however, is an oversimplification as these shallow inshore regions are areas of considerable environmental variability and instability that may potentially perturb the developmental process (Augustyn et al. 1994; Oosthuizen et al. 2002; Steer et al. 2002; Chapter three).

Given the sub-annual life cycle typical of loliginid squid, it is imperative that hatching success is maximised to ensure the production of the subsequent generation. To lower the risk of embryo mortality, eggs are well protected from predation by a series of mucous layers and are laid over extended temporal and spatial scales. Females therefore spread the risk of juvenile mortality by not putting all the eggs in one basket (O'Dor 1998). Mortality rates and developmental abnormality are highly variable in wild *Sepioteuthis australis* embryos, ranging from 4 to 20% (Gowland et al. 2002b; Steer et al. 2002; Chapter three). Fluctuating temperatures are considered the major factor responsible for developmental error in the laboratory (Boletzky and Hanlon 1983; Hanlon 1990; Gowland et al. 2002a). However, fluctuations in the order of 1-2 °C day^{-1} were not sufficient to promote embryo mortality in the field (Steer et al. 2002; Chapter three). Therefore, it was hypothesised that variation in embryo mortality was a function of other unmeasured environmental parameters, or the micro-environment within the egg mass (Steer et al. 2002; Chapter three).

The nature of the egg mass may have inherent limitations associated with it. For example, embryos enclosed within egg capsules may be constrained by the morphology of the egg mass and the availability of ambient oxygen (Cronin and Seymour 2000). A number of studies suggest that natural aggregations of embryos approach or exceed limits for supply of oxygen resulting in retarded, or dead embryos located in the centre of the egg mass (Strathmann and Chaffee 1984; Cohen and Strathmann 1996). To further complicate this, egg strands become fouled, especially during the later stages of development, and it has also been suggested that respiration by these fouling organisms can also contribute to hypoxic conditions (Benkendorff 1999). The relative position of *S. australis* embryos within an egg strand and presence of biofouling organisms on the strands' surface plays a significant role in embryo mortality (Steer et al. 2002; Chapter three). However, it is not understood what role egg mass size, position of the embryo within the mass, the type of substrate it is attached to, and the degree of biological fouling has on embryo mortality.

The southern calamary *Sepioteuthis australis* egg masses are typical of loliginid squid, where females' package three to nine eggs within a protective, digitate strand. Each female is capable of laying a series of egg strands, individually attaching each one to a common holdfast to form an egg mass. Numerous females can contribute to a single egg mass (Jantzen and Havenhand 2002), and therefore the resultant mass may be >600 strands (Moltschaniwskyj and Pecl 2003). Using a combination of field surveys and laboratory experiments this study investigated the effect of egg mass size, the substrate upon which it is attached, the position of the embryo within the mass, and the degree of biofouling on embryo mortality. In addition, extensive spatial egg surveys were carried out to determine whether egg mass density influences the size of aggregated egg masses.

4.3. Materials and Methods

Monthly *Sepioteuthis australis* egg surveys, extending from October 2001 to February 2002, were carried out on the eastern and south eastern coasts of Tasmania (Fig 4.1). In each region 5-17 shallow (<10m) sites, displaying similar

characteristics to known calamary spawning areas (ie seagrass beds, macroalgal forests associated with rock reef habitats, and areas of patchy vegetation) were targeted. To assess the intensity of calamary spawning activity in the region, 20 min timed swims were carried out at each site, during which the substrate was searched by two SCUBA divers. Given egg masses were of varying size the number of egg strands was considered a better indicator of spawning intensity rather than the number of egg masses. However, most egg masses were made up of more than 50 strands and it was not logistically possible to count the strands in each egg mass while underwater. The length of an egg mass, as measured from its attachment point to the tip of the terminal strand, was therefore recorded and the number of strands subsequently calculated from a predictive regression equation established by Moltschaniwskyj et al. (2002). When an egg mass with less than 20 strands was encountered, the number of strands was counted directly. Using the search time data it was later possible to determine relative egg density of the area.

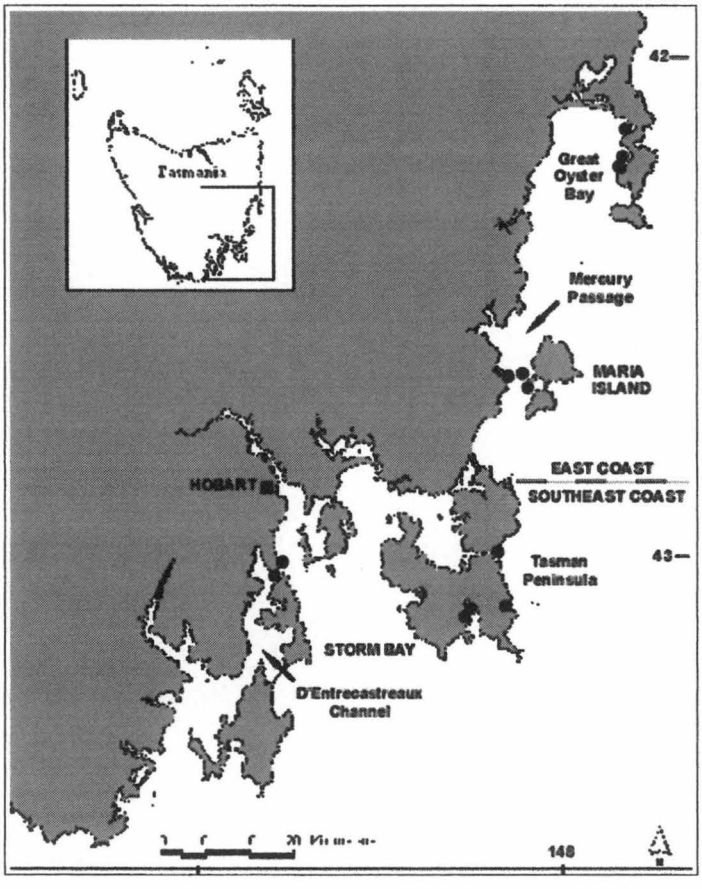


Figure 4.1. Map of the east coast of Tasmania, divided in to east and southeast regions indicating areas (black circles) where *S. australis* eggs were collected.

Samples were collected from egg masses containing late stage embryos, i.e., those which had developed passed stage 20 (see Steer et al. 2003; Chapter two). Each sample consisted of six egg strands; three strands completely unfouled and three that had some degree of biofouling. A pilot study investigating patterns of biofouling in calamary egg masses indicated that the terminal ends of internally located egg strands supported significantly more fouling ($F = 5.79$, $df = 1$, $P = 0.02$) than peripheral strands. Consequently, in this study fouled strands were selected from the interior of the egg mass. Percentage cover of biofouling on each strand was estimated (<25%, 26-50%, 51-75%, and 76-100%). The substrate type upon which the egg masses was attached was recorded and broadly categorised as either soft-sediment vegetation (*Amphibolis antarctica*, *Heterozostera tasmanica* sp., *Caulerpa* sp.), macroalgae (*Ecklonia* sp., *Macrocystis pyrifera*, *Sargassum* sp.), or other (unattached or partially buried in sand). At each site samples were collected from 4-15 egg masses.

Embryos were dissected from each strand within 8 hours of collection and assigned a developmental stage according to the criteria described by Steer et al (2003) (Chapter two). The position of each embryo within the egg strand was also recorded; position 1 identified the egg located at the fixed/proximal end of the strand and progressing consecutively to the free/distal end of the strand. Eggs that were unfertilised, dead, or undergoing abnormal development were scored as "dead".

To determine how the number of strands in a mass affected mortality rates three large (>500 strands) recently laid egg masses (<stage 10), free from biological fouling, were collected from Mercury Passage and transported to aquarium facilities. Each mass was divided into six clusters consisting of 5, 25, 50, 100, 200, and 200+ strands and each cluster was suspended by a length of nylon thread in one of six 100-L tubs connected to a closed 1400-L, recirculating system maintained at ambient temperature. All eggs were completely submerged during handling to reduce the risk of damage due to air exposure. Flow rates in each tub were adjusted so each egg mass was gently agitated ensuring that they were not suspended in stagnant water. Gentle surface aeration was provided and water quality monitored three times a week. A 12:12 light:dark regime was set. Eggs were destructively sampled pre-

hatching and masses containing five strands were completely dissected. For egg masses with more than five strands, 10 strands were sampled, five from the interior of the mass and five from the periphery. The within strand position and status (i.e., dead or alive) of the embryo was recorded.

4.3.1. Statistical Analysis

Comparisons of egg mass density and sizes between regions and among months were carried out using ANOVA and chi-square analysis. For ANOVA, residual plots were used to assess equality of variances and where necessary data were square-root transformed.

Embryo mortality frequency across a three-way contingency table incorporating substrate type, degree of fouling, and position of the embryo within an egg strand as the main factors was analysed using log-linear analysis. The incorporation of month and regional factors were avoided due to missing, low, or zero cell frequencies (Quinn and Keough 2002). In a further attempt to collapse the contingency table only egg strands containing five embryos were used (mean ± se, 5.24 ± 0.03, n = 1002 strands), however data from longer strands containing >5 embryos were graphically presented.

Log-linear analysis was also used to compare the frequency of embryo mortality in laboratory reared eggs as a function of mass size, position within the mass (internal or peripheral), and position within the egg strand. Egg masses containing five egg strands were not included in the analysis due to difficulties in discriminating between internally located and peripheral strands, however, data were still graphically presented.

4.4. RESULTS

A total of 560 calamary egg masses were surveyed from October 2001 to February 2002 and samples were collected from 166 egg masses. Spawning intensity, as measured from egg density, differed between regions ($F = 4.59$, $df = 1, 102$, $P = 0.035$), but not among months ($F = 0.77$, $df = 5, 102$, $P = 0.577$). The

density of egg masses was approximately three times greater on the east coast (0.33 ± 0.05 egg masses per 20 m^2, n = 84) than in the south east (0.12 ± 0.09, n = 30). A Majority of *Sepioteuthis australis* egg masses sampled (59%) were associated with shallow water (<4m) soft-sediment vegetation; 27.4% were attached to the holdfasts of macroalgal species in depths >4 m; while the remaining masses (12.7%) were either dislodged or partly buried in sandy sediments. Two egg masses were found attached to exposed tubeworm casts. The seagrass *Amphibolis antarctica* is the dominant shallow seagrass in non-estuarine waters on the east coast of Tasmania, extending from Great Oyster Bay to Mercury Passage and as a result was the preferred substrate for spawning calamary in these areas. Species of seagrass were observed to gradually change as sampling moved south, with calamary eggs attached to *Heterozostera tasmanica* together with sparse cover of *Amphibolis* on the Tasman Peninsula, to purely *H. tasmanica* in the D'Entrecastreaux Channel area. The soft-sediment associated macroalgae, *Caulerpa* sp. also became more dominant further south. The macroalgal species *Macrocystis pyrifera, Ecklonia* sp., and *Sargassum* sp. were the main spawning substrates in deeper waters (>4m).

The size of the egg masses ranged from 3-1241 strands on the east coast and 10-619 strands in the south. Single strands containing viable eggs were occasionally found (<1.0% of observations) attached at the end of seagrass blades. There was no significant difference in the size frequency distribution of the egg masses between the two regions (χ^2 = 3.45, df = 4, P = 0.49). Most of the egg masses at both locations consisted of 200-299 strands (Fig 4.2). The smallest egg masses were generally secured to soft-sediment vegetation whereas the largest masses were attached to the holdfasts of macroalgae, unattached or partially buried in sand (F = 6.67, df = 2, 154, P = 0.002, Fig 3). A weak positive correlation between egg mass size and number of dead embryos was evident (Pearson's correlation, r = 0.39, n = 160, p <0.001), with approximately 13-51% of the embryos examined in masses >615 strands either developing abnormally or dead.

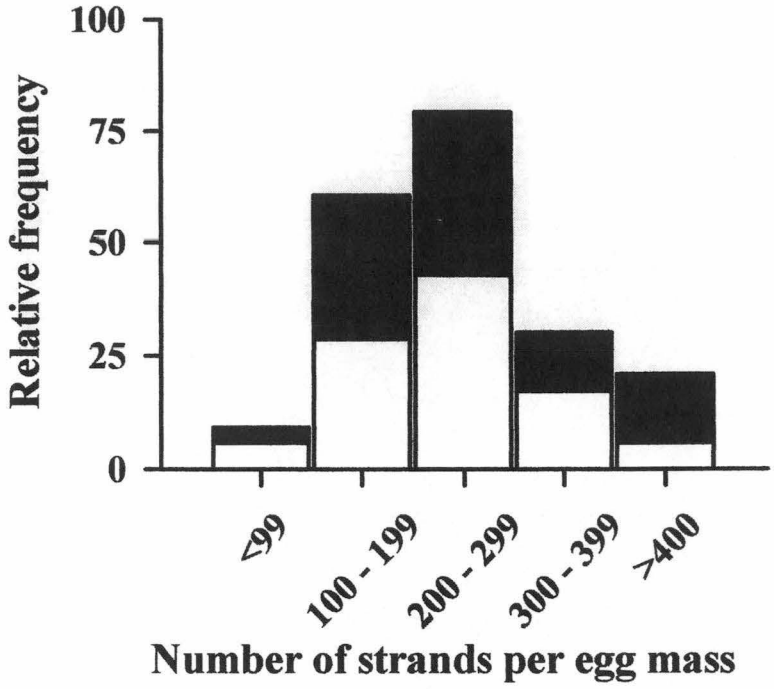


Figure 4.2. The relative size frequency of *S. australis* egg masses measured as number of egg strands per mass. Black bars represent eggs collected from the east and white bars from the south east coasts of Tasmania.

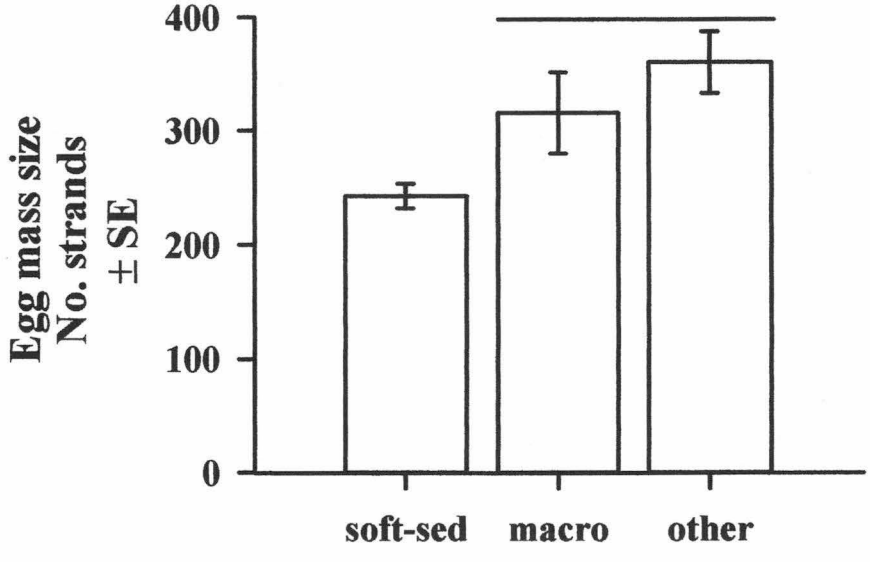


Figure 4.3. The average *S. australis* egg mass size ± standard error attached to different substrate types; soft-sediment vegetation, macroalgae and other (unattached, partially buried). Horizontal bars overlap means which are not significantly different as determined by a Hochberg GT2 post hoc test.

A total of 5249 individual embryos were sampled. Due to the nature of the field surveys and the patchiness of calamary spawning there were some sampling periods that yielded very few egg masses and periods where eggs were only attached to a particular substrate. As a result there were a few gaps in the data series. Nevertheless, mortality rates were variable throughout the spawning season, ranging between 2–25%. Unattached egg masses contained significantly more dead embryos than attached masses (G^2 = 84.03, df = 2, p <0.001, Fig 4.4). Embryos located at position 1 in unattached masses had the highest mortality at 32%. Mortality rates dropped sequentially along the egg strand until position 5 (Fig 4.5). Mortality rates in attached masses followed a similar trend, but were consistently 50% lower, consequently no interaction between position of the embryo within the strand and substrate type was detected (G^2 = 5.03, df = 10, p = 0.89). Although the analysis did not include embryos collected from terminal positions 6 and 7, mortality rates in unattached masses dramatically increased to 30%, whereas rates in attached masses remained below 5% (Fig 4.5).

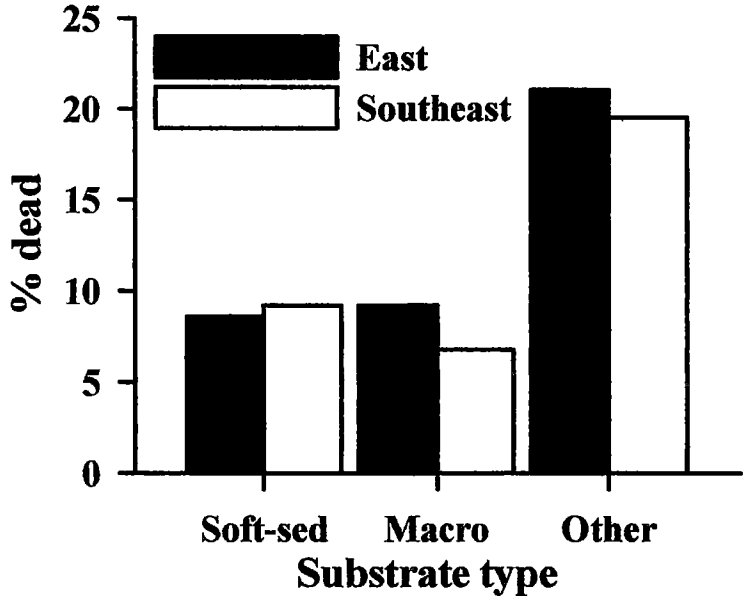


Figure 4.4. Proportion of dead *S. australis* embryos within masses associated with different substrates; soft-sediment vegetation, macroalgae and other (unattached, partially buried) located on the east and south east coast of Tasmania.

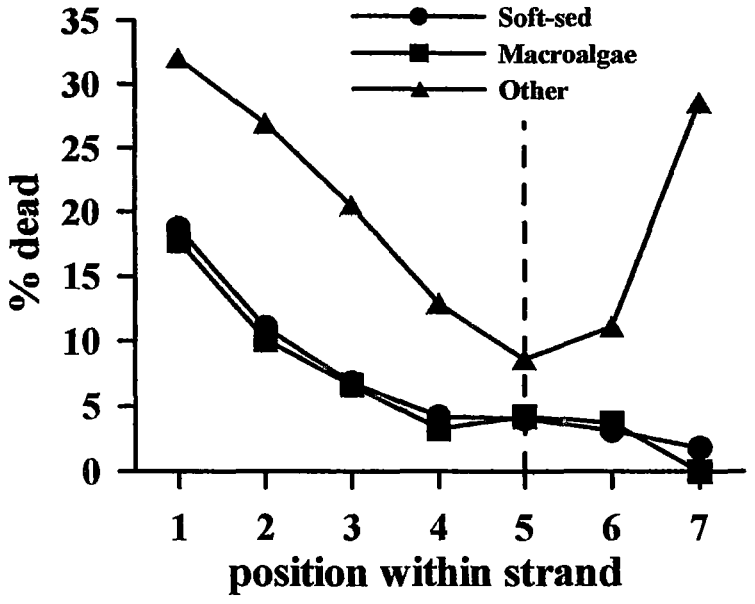


Figure 4.5. Proportion of dead *S. australis* embryos within individual egg strands collected from different substrates. Position 1 represents embryos that are located at the base/proximal end of the egg strand whereas position 7 represents embryos at the end of the egg strand. Vertical dashed line at position 5 indicates the boundary of statistical analysis. Due to poor replication of longer strands only egg strands containing 5 embryos were included in the analysis.

A significant interaction between degree of fouling and egg position was detected (G^2 = 26.36, df = 15, P = 0.03). At the proximal end of the egg strand embryos in relatively unfouled (0-25% fouled) strands displayed the highest mortality rates (Fig 4.6). An obvious inverse relationship existed between relative mortality and degree of fouling at the proximal position as the most heavily fouled eggs displayed mortality rates more than 50% lower than unfouled eggs (Fig. 4.6). This trend, between fouled and unfouled strands continued mid-way along the strand with mortality rates dropping sequentially (Fig 4.6). However, at the terminal end of the strand mortality rates increase to ~15% in heavily fouled egg strands (50-100% fouled) and 0% in relatively unfouled strands.

Strong position effects on embryo mortality with respect to the location of the egg strand within an egg mass and the position of the embryo within an individual

egg strand were detected in laboratory reared eggs. Embryos developing at the proximal end of the strand displayed consistently higher mortality rates compared with their distal siblings, similar to that observed in field collected eggs. This trend was significantly exaggerated ($G = 79.25$, df = 7, $P < 0.001$) in internal egg strands where 95% of position 1 embryos were either developing abnormally or were dead compared to 43% dead in peripheral strands (Fig 4.7). Embryo mortality within internal egg strands decreased sequentially along the strand to 31% at position 7. Mortality rates in peripheral strands, however, remained below 10% from position 4 onwards.

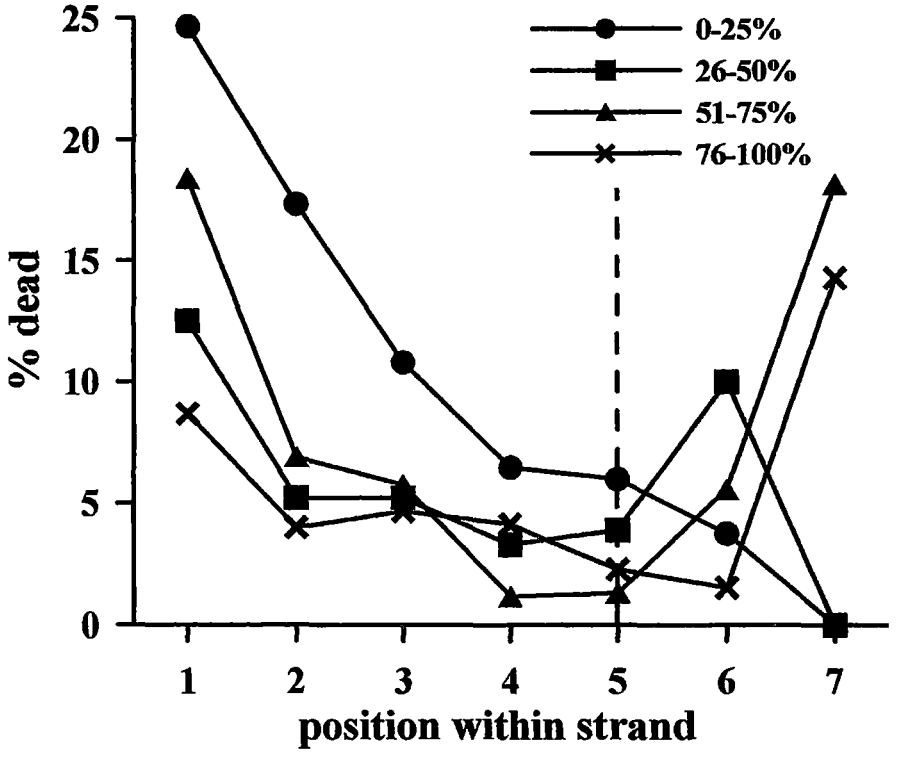


Figure 4.6. Proportion of dead *S. australis* embryos within individual egg strands supporting varying degrees of fouling. Position 1 represents embryos that are located at the base/proximal end of the egg strand whereas position 7 represents embryos at the end of the egg strand. Vertical dashed line at position 5 indicated the boundary of statistical analysis. Due to poor replication of longer strands only egg strands containing 5 embryos were included in the analysis.

In the experimental adjustment of egg mass size, increasing mass size had significantly influenced embryo mortality ($G = 73.93$, df = 4, $P < 0.001$). Egg masses containing ≥100 egg strands had 30% more dead embryos than those containing ≤50 egg strands (Fig 4.8). These mortality estimates were greatly inflated by extremely high incidences of dead embryos (approx. 90%) within internally located strands. These strands also displayed distinct signs of deterioration and decomposition compromising the integrity of the entire strand, whereas strands on the periphery of the mass were relatively unaffected. Embryos developing in the peripheral strands consistently displayed mortality rates lower than 25%, regardless of egg mass density. Although egg masses containing five strands were not included in the analysis, they yielded comparable results with the peripheral strands displaying 16% mortality.

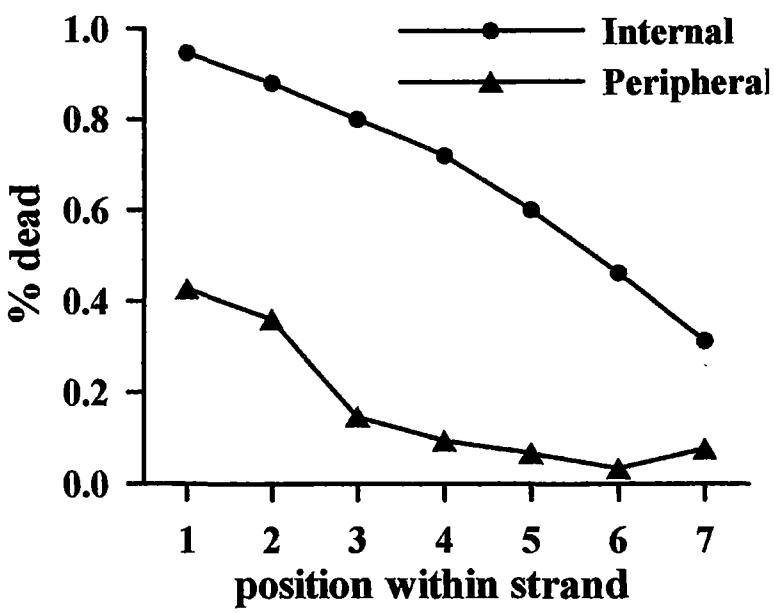


Figure 4.7. Proportion of dead *S. australis* embryos within individual egg strands collected from the interior and periphery of an aggregated egg mass. Position 1 represents embryos that are located at the base/proximal end of the egg strand whereas position 7 represents embryos at the end of the egg strand.

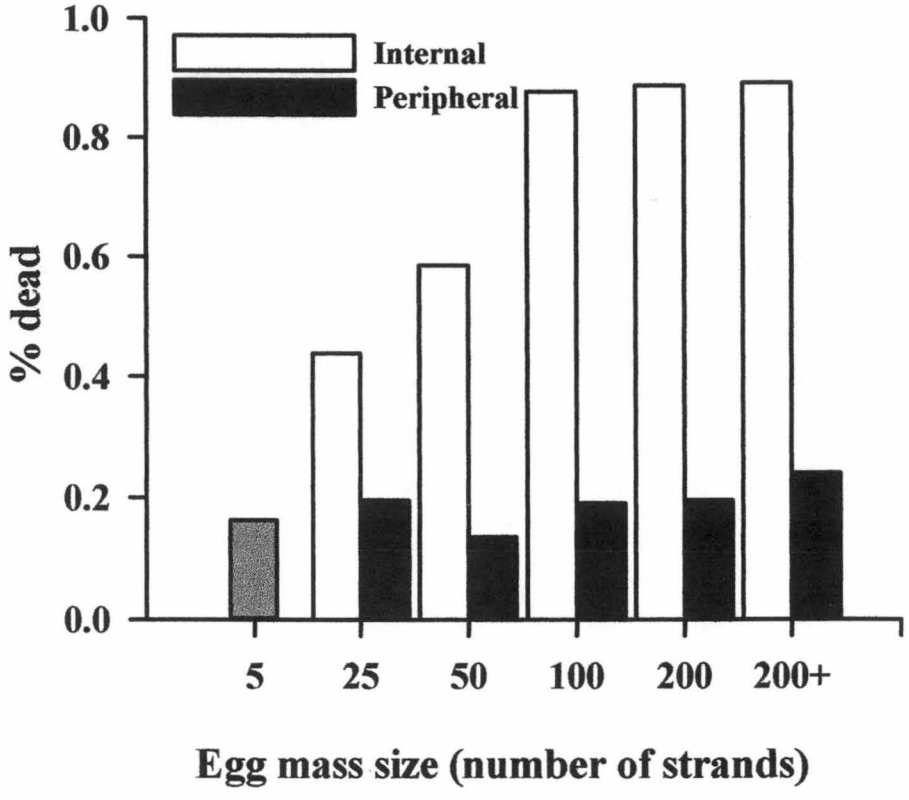


Figure 4.8. Proportion of dead *S. australis* embryos collected from the interior and periphery of different size egg masses. Grey bar represents data not included in the analysis.

4.5. DISCUSSION

The relative position of *Sepioteuthis australis* embryos within an aggregated egg mass determines it chances of survival. Embryos located around the attachment point of the egg strand, within the interior of the mass, or in close contact with the substrate are more likely to die (due to the inability to exchange gases effectively (Strathmann and Chaffee 1984)). This susceptibility to mortality, however, is directly related to the density of the egg mass, with larger masses containing proportionately more dead embryos, a trend that was consistent in both laboratory and field investigations. Such large masses may increase embryo mortality rates for two reasons. Firstly there is increased physical stress placed on internally located embryos as a function of overcrowding. Secondly there is an increased risk of physical detachment of the egg mass from its substrate.

Larger egg masses are more prone to detachment purely due to their large surface area and susceptibility to current action (Sauer et al. 1993). This study partially supports this, as larger masses (>300 egg strands) were unattached, partially buried or securely attached to robust macroalgae holdfasts. The risk of detachment would obviously depend on the strength of the currents and the relative strength of substratcs' foundation. This would explain why some larger masses were found attached to the strong foundation of macroalgae holdfasts and not frailer seagrass stipes. In addition, unattached egg masses were frequently found attached to uprooted or remnant pieces of vegetation suggesting that they had been physically dislodged from their original position. The loss of eggs due to storm activity has been documented (Sauer et al. 1993; Moltschaniwskyj and Pecl 2003) but, the fate of the embryos has never been directly assessed.

Dislodged egg masses have distinct disadvantages, for example, they are in constant physical contact with the benthos, run the risk of being buried, and depending on the currents can be transported to unfavourable environments. Generally in calm conditions, the physical weight of the egg mass and gentle swell motion sweeps the mass into crater-like depressions as described by Sauer et al. (1993) for *L. vulgaris reynaudii.* Being confined in a sandy depression, and in direct physical contact with the benthos, potentially reduces the embryos ability to exchange gases effectively. Embryos of the pond snail (*Lymnaea stagnalis*) in direct contact with the substrate displayed slower development, suggesting that surface exposure and not neighbouring eggs is a limiting factor (Marois and Croll 1991). To further support this, laboratory reared *S. australis* eggs in direct contact with the aquarium walls were often found to contain dead embryos, which resulted in the entire strand dying (Steer, pers obs). These strands exhibited similar signs of deterioration and decomposition observed in internally located, laboratory reared strands in this study. Although this is more frequently observed in an unnatural laboratory environment, there was evidence of egg decomposition in unattached egg masses in the field, potentially contributing to elevated mortality rates. Although it is unclear, it can be suggested that areas of decomposition within an aggregated mass 'infects' neighbouring embryos consequently compromising their survival.

Strong swells can transport unattached egg masses across large distances, and it is not uncommon to see loliginid egg masses washed ashore after severe weather (Boletzky 1986). Although the direct consequence of the physical rolling of the unattached mass along the benthos on the developing embryo is unknown, it is likely to be detrimental, especially if the mass is washed ashore for extended periods. A higher incidence of dead embryos was found in the terminal ends of unattached masses in comparison to attached masses. It is possible that embryos in terminal positions 6 and 7 have died as a result of being bumped and tumbled across the substrate. This result, however, must be interpreted with caution as strands consisting of 6+ embryos represented <8.8% of the total observations. Nevertheless, the effect of rolling egg masses and potential abrasion of the sand on the developing embryos, particularly those in direct contact with the benthos, requires further investigation.

Embryo mortality rates were highest in unfouled egg strands. This may be related to the physical structure of the egg mass, with biofoulers colonising areas that are conducive to growth, respiration and photosynthesis. Egg strands deep within the egg mass, shaded by neighbouring egg strands, or in direct contact with the substrate, would not provide a suitable substrate for fouling. Therefore, it is not surprising to see that embryos in these strands displayed the highest levels of mortality. It is therefore likely that healthy *S. australis* eggs support epiphytic growth rather than epiphytic growth ensuring healthy embryos (Gowland et al. 2002b).

Colonisation of biofoulers on *S. autralis* egg strands is suggested to be delayed by a chemical defence allowing embryos sufficient time to hatch before fouling has any effect (Benkendorff 1999). This, however, may not always be the case as depending on conditions fouling organisms may rapidly colonise egg strands before juveniles hatch. In this study the terminal ends of the strands (position 7) that are heavily fouled (>50%) contain proportionately more dead embryos than those located down the strand. It is possible that fouling organisms have rapidly colonised the strand's surface to a point where the embryo is effectively trapped within the egg strand. To further support this, dead mature hatchlings were occasionally observed to have partially penetrated the fouled egg strand suggesting that they had become stuck and died in the hatching process. It is assumed that Choe (1966) made similar

observations in loliginid, sepiolid and sepiid eggs as it was found that large quantities of diatoms and green algae colonising the eggs were responsible for 'unsatisfactory' hatching.

Egg mass size in communal spawners such as *S. australis,* is not determined by the individual and potentially depends on how many females are contributing to the mass. In some species tens to hundreds of females may contribute to existing egg masses subsequently increasing their overall diameter to >3m (≈1000-10,000 strands) (eg *Loligo opalescens* McGowan 1954; *L. pealei* Griswold and Prezioso 1981; *L. vulgaris reynaudii* Sauer et al. 1992). Such large egg masses have not been observed in *S. australis* with the largest egg mass recorded in this study consisting of 1240 egg strands, and the majority in the 200-299 size class. Given a 3-fold difference in egg production between the regions, but no difference in the frequency distribution of egg mass size, it is unlikely that egg mass size was a function of spawning intensity. This suggests that other factors, such as spawning behaviour or substrate type potentially determines egg mass size. Factors determining when and where females deposit their eggs may have underlying evolutionary significance, raising interesting questions such as; why do some females contribute to existing egg masses where others start new masses? What are the benefits, in terms of hatching success, of these two methods? Adding strands to the periphery of existing masses, where conditions for development are favourable, would theoretically maximise hatching success whilst also competing against other embryos. Contributing to an existing egg mass will ensure that more juveniles hatch simultaneously, therefore reducing the individual's chances of being predated upon. The decision to begin a new egg mass may relate to its theoretical critical size (Sauer et al. 1993; Strathmann and Strathmann 1995). For example; the benefits of laying eggs on a stable substrate, elevated from the benthos may out weigh contributing eggs to a large egg mass prone to being dislodged by wave action. The behavioural and social aspects in spawning loliginids are complex (Sauer et al. 1997; Hanlon 1998; Jantzen and Havenhand 2003) and further work is required to specifically address these questions, unravelling whether trade-offs exist in spawning site preferences and competitive interactions.

The size of the egg mass, the position of the embryo within the mass and the substrate to which it is attached are important factors in determining *Sepioteuthis australis* hatching success. Although interiorly located embryos display consistently higher mortality rates regardless of substrate, the risk is almost doubled in larger masses, particularly those that have been detached from low relief vegetation. Embryos situated around the periphery of the mass or are laid in small discrete masses, attached to solid substrates are therefore more likely to survive, providing the environmental conditions are favourable. In general, *S. australis* lays relatively discrete egg masses with approximately 10% of all embryos examined in this study lost to mortality. These results raise interesting questions relating to the spawning behaviour and viability of eggs laid by squid species that contribute to large communal beds on potentially unstable, sandy substrates.

CHAPTER FIVE

THE ROLE OF TEMPERATURE AND MATERNAL RATION IN EMBRYO SURVIVAL: USING THE DUMPLING SQUID *EUPRYMNA TASMANICA* AS A MODEL

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5.1. ABSTRACT

Using a 'model' sepiolid *Euprymna tasmanica* this study investigated the role of maternal nutritional and thermal history on egg quality and subsequent embryo survival. As *E. tasmanica* produces multiple batches of eggs it was possible to track egg quality and hatching success over successive spawning episodes. A two factor orthogonal experimental design, involving two feeding levels (high and low rations) and two temperatures (summer and winter), was implemented with half of the replicates used to explore embryonic development and the remaining half examining egg-yolk quality via fatty acid analysis. Differences in reproductive output and embryo mortality were largely attributed to maternal ration and not temperature. Females maintained on low ration produced smaller clutches, consisting of smaller eggs and exhibiting higher embryo mortality rates than high ration females. Both batch fecundity and relative hatching success declined over successive clutches. Lipid content was also significantly lower in low ration females, however the relative quality in terms of lipid and fatty acid constituents was maintained regardless of treatment and spawning frequency. It is suggested that elevated embryo mortality rates in eggs spawned by low fed females was a function of insufficient maternally derived yolk resources to fuel embryogenesis. Both maternal nutritional and reproductive history were important determinates for offspring survival, which potentially has significant effects on the magnitude of recruitment events in squid populations.

5.2. INTRODUCTION

Definitive links between size, age, and condition of mature females and embryo/larval survival have been identified in a diverse range of marine taxa (Bayne et al. 1975; Laine and Rajasilta 1999; Keckeis et al. 2000; Jimmy et al., 2002; McCormick 1999; 2003). Recent studies suggest that maternal nutritional history has a large influence on embryonic development and offspring competency through the sequestering and provisioning of yolk resources (Laine and Rajasilta 1999; McCormick 2003). Maternal temperature effects, through interactions with food availability, are also considered important determinates of offspring size (McKee and

Ebert 1996) and potentially larval competency. As a result, fluctuations in environmental conditions, particularly food availability, experienced during the females' reproductive season can have significant flow on effects to the subsequent population structure (Kerrigan 1997).

Non-overlapping, sub-annual cephalopod populations exhibit considerable spatial and temporal variation in recruitment (Pierce and Boyle 2003). This variation is expected to be strongly affected by environmental conditions, particularly affecting the early life stages (Boyle and Boletzky 1996; Pierce and Boyle 2003). Currently little is known about processes of early mortality, however, quantitative data exists for the southern calamary *Sepioteuthis australis* indicating that natural embryo mortality rates vary considerably over the spawning season (Steer et al., 2002; Chapter three; Chapter four). *Sepioteuthis australis* spawns multiple clutches over a relatively short spawning period and exhibits substantial variation in the size and number of ovulated eggs (Jackson and Pecl 2003). Lipid quantity varies with egg size in the cuttlefish *Sepia officinalis* suggesting that differences in egg quality may exist (Bouchaud and Galois 1990). Significant spatial differences in egg quality, in terms of lipid content also occurs (Boyle et al. 2001). The lipid requirements for correct cephalopod development is not understood, however phospholipids and long chained polyunsaturated fatty acids, particularly EPA (20:5ω3) and DHA (22:6ω3) are likely to be essential constituents (Bouchaud and Galois 1990; Navarro and Villanueva 2000, 2003; Boyle et al. 2001). Therefore, it would be beneficial to investigate whether spatial and temporal fluctuations in embryo mortality are linked to egg quality spawned by females from different nutritional and thermal environments.

Attributing *Sepioteuthis australis* embryo mortality to maternal nutritional and thermal history in the field is logistically challenging. This is largely due to the difficulties associated with linking females with spawned eggs and ascertaining the females' history. Furthermore, the relative condition of the female may be confounded by her reproductive status. Serial spawning females exhibit no clear physiological record of previous spawning events (Sauer et al. 1999; Moltschaniwskyj and Semmens 2000), therefore it is virtually impossible to determine whether spawned eggs are from the first or last spawning episode. In the

serial spawning Atlantic cod, *Gadus morhua*, egg size and quality varies throughout successive spawning events (Chambers and Waiwood 1996; Ouellet et al. 2001). This variation is attributed to changes in female condition over the spawning season with past reproductive investments influencing future investments (Kjesbu et al. 1996). Therefore, when determining the flow on effects of maternal condition, through to egg quality and subsequent embryo survival in multiple spawners, the females' reproductive history must be considered.

Due to the logistical difficulties associated with maintaining large, highly mobile, cephalopods in captivity (Hanlon 1990) and assessing links between maternal condition and hatching success in field populations, it is necessary to use a 'model' species conducive to manipulative experimentation. The southern dumpling squid *Euprymna tasmanica* (Sepiolidae) was chosen in this study, as it is a small multiple spawning cephalopod that is easily collected and reared in captivity. Using this species this study aimed to describe the relationship between the nutritional and thermal environment in which females are exposed and the quantity and quality of the eggs and embryos produced over successive clutches.

5.3. Materials and Methods

5.3.1. Experimental design

To determine the effects of water temperature and level of feeding on reproductive parameters 36 females were randomly allocated to one of four treatment combinations (18°C – high feeding, 18°C – low feeding, 11°C – high feeding, 11°C – low feeding). All animals used in the experiment were collected from sand flats in northern Tasmania during night low tides. Due to space constraints in the laboratory the 11°C treatment was run during the austral winter (2001) when ambient water temperature when the animals were collected was ~11°C and the 18°C treatment was run in summer (2002) when the temperature was ~18°C. Males and females were separated upon collection to avoid mating and transported to the aquatic facilities located within the School of Aquaculture (University of Tasmania). Upon arrival females were housed in individual (300 x 150 x 100 mm) plastic aquaria attached to a closed 1400 L recirculation system. Each aquarium contained a half piece of PVC

piping for shelter and a secure lid. Water quality was monitored three times a week and maintained within these levels; salinity 34-36‰, NO_2 <0.1 mg/L, NO_3 <10.0 mg/L, NH_4 <0.25 mg/L. All males were collectively housed in a 100-L tank. The prey species used during the experiment were mysid shrimp *Tenagomysis tasmaniae*, *Paramesopodosis rufa*, and *Anisomysis mixta australis*. For 1-2 weeks prior to the start of the experiment females were fed ad libitum to allow time for individuals to habituate to their new surroundings.

Once habituated, females were randomly allocated to either a high or low feeding regime, where high fed females were fed daily and low fed females fed 2-3 times a week. After two-weeks on the treatment feeding regimes females were weighed and a male of known weight was introduced to each female and removed after mating was observed. Females were left in isolation until a clutch of eggs had been laid. The egg clutches of four females in each feeding-temperature combination were removed within twelve hours of being laid. The number, total egg wet weight, and egg capsule wet weight (= total egg wet weight minus protective layer wet weight), and individual egg volume (egg volume = (4/3) π r d, where r = radius of the longest axis and d = the diameter of the egg capsule) was recorded prior to being frozen (-86°C) for lipid analysis. Egg clutches from the remaining four females were left in situ and the time to hatching, hatching success, and size (dorsal mantle length ML and weight) of the hatchlings was recorded. Embryos reared at 11°C were destructively sampled before hatching due the extremely slow development time (~20 weeks), and the need to run the 18°C treatment before the end of summer. Eggs that were unfertilised, had ceased development or were undergoing abnormal development were considered 'dead', as they were unlikely to successfully hatch. Males were reintroduced 2-5 days after a female laid a clutch and the entire process repeated for three successive clutches. To minimise the influence of paternity on embryo growth and survival (Shaw and Boyle 1997) only four males were used within each temperature regime, with each male used to mate a single female in each of the feeding levels. All interactions and manipulations with animals were made during daylight hours.

5.3.2. Lipid and Fatty Acid Analysis

Due to small amounts of dry material the entire freeze-dried egg mass (minus multiple protective layers) was ground up in a mortar and pestle before extraction. Total lipid was extracted from freeze dried eggs using a modified one-phase CH_3OH-$CHCl_3$-H_2O (2:1:0.8 v/v/v) Bligh and Dyer (1959) extraction. Phases were separated after 18 hours of extraction under low light by the addition of CH_3OH and H_2O (1:1 v/v) and 0.1 g NaCl. Lipids were recovered in the lower CH_3OH phase and the solvent was removed under rotary vacuum leaving the total lipid extract (TLE). TLE was weighed to obtain a percentage of the dry weight of the egg mass. All samples were made up to 1500µl in chloroform and stored at -20°C.

An aliquot (~1µml) of the TLE was analysed with an Iatroscan (model TH-10 MKII, Iatron, Japan) thin layer chromatography-flame ionisation detector (TLC-FID) to determine the proportion of major lipid classes. A polar solvent system (60:17:0.2 v/v/v ratio of C_4H_{10}:$(C_2H_5)_2O$:CH_3COOH) was use to resolve lipid classes after samples were applied to silica gel SII chromarods (5 µL particle size) using 1 µL micropipettes. The FID was calibrated for each compound class. Computer software MACLAB Chart v3.5 and Peaks v1.4 were used to quantify areas of lipid class peaks.

A 300µl aliquot was taken from the TLE and trans-esterified at 80°C for two hours in a CH_3OH:$CHCl_3$:HCL (10:1:1 v/v/v) mixture to produce fatty acid methyl esters (FAME). FAMEs were partitioned by the addition of water and extracted with C_4H_{10}: $CHCl_3$ (4:1 v/v). Samples were centrifuged at 1500 rpm for three minutes and the top phase consisting of FAMEs was retained, reduced under nitrogen and stored at -20°C. An internal standard (19:0) of known concentration was added.

Fatty acid components were determined by gas chromatography using a Hewlett Packard 5890 series II gas chromatograph (GC) and a HP 5971A mass selective detector (MSD). A 60m x 0.25mm internal diameter HP-1MS column (0.25 µm film thickness) was used. GC and MSD operating conditions were similar to those described by Nichols et al. (1994). Peaks were quantified using ChemStation software and identified by comparison of component mass spectra and

retention times with a well-characterised menhadin marine oil standard. Both fatty acids and lipid classes were presented as a mean percent (± standard deviation) of sample per treatment.

5.3.3. Statistical Analysis

A two-way analysis of variance (ANOVA) was used to compare parameters of interest with feeding level and temperature as the orthogonal factors. Consecutive clutches produced by females was treated as repeated measures data and analysed using a univariate split-plot ANOVA. Due to the nature of the experiment the design became unavoidably unbalanced as many females senesced before laying three clutches. Therefore, Type III sum of squares were calculated using unweighted marginal means which are not influenced by the sample sizes in each cell (Quinn and Keough 2002). In some cases the third clutch was omitted from the analysis, however means were still presented.

Mixed model nested ANOVA was used to determine differences in average egg volume and hatchling size as a function of temperature and ration with females as a nested term within treatment combinations. Due to reduced replication for successive clutches only parameters measured from the first clutch were included in the analysis. Data was transformed where necessary to deal with violations of assumptions of ANOVA.

5.4. RESULTS

Each egg was individually laid and coated in numerous semi-transparent, gelatinous layers and a tougher, opaque-orange outer flexible layer. Eggs were typically individually attached to a common substrate either abutting, on top of, or in close proximity to each other to form discrete egg masses arranged in an amorphous structure. Across all the treatments 44.7% of the females deposited three clutches. The number of eggs deposited in a clutch at any one time was highly variable ranging from three to 107 eggs and there was a significant decrease in the number of eggs in successive clutches (Fig 5.1, Table 5.1). However, the rate of decline in the size of successive clutches was not significantly affected by either ration level or

temperature (Table 5.1). However, across all clutches the total number of eggs laid was affected by ration, but not temperature (Table 5.1). Females maintained on high rations consistently produced ~60% more eggs than those maintained on low ration. This was consistent across successive clutches (Bonferroni adjusted two tailed *t*-tests, df = 36, Clutch 1; t = -5.02, P > 0.001. Clutch 2; t = -3.28, P = 0.001; Clutch 3; t = -2.539, P = 0.016).

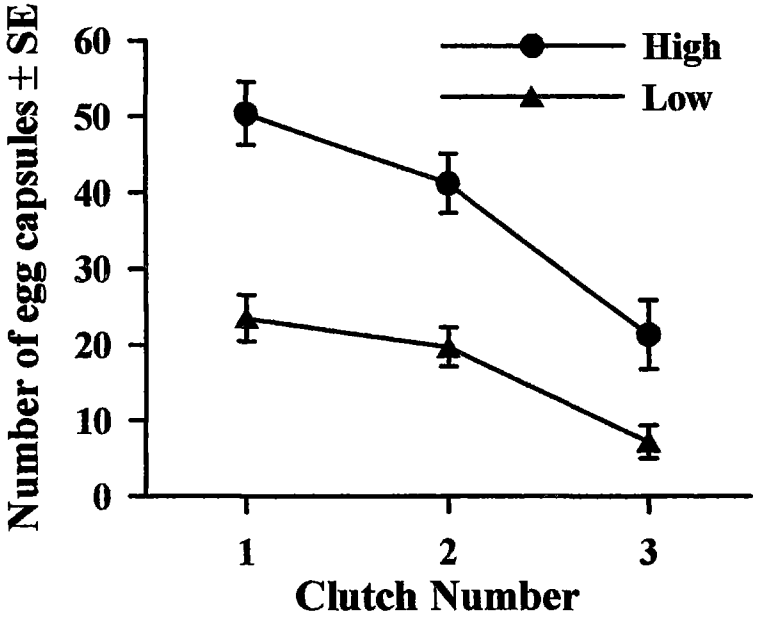


Figure 5.1. Changes in batch fecundity (average number of egg capsules ± standard error) over successive clutches for mature *Euprymna tasmanica* maintained at high and low feeding regimes.

There was no evidence that the size of the female was correlated with total reproductive output (Pearson's two-tailed, r = -0.308, n = 20, P = 0.19). Likewise there was no correlation between female weight and average egg volume (r = 0.29, n = 16, P = 0.25). Egg size was significantly affected by ration (F = 11.07, df = 1,12, P<0.01), but not temperature (F = 2.12, df = 1,12, P = 0.17). Eggs spawned in the initial clutch by females maintained on the higher ration were consistently larger than those maintained on the lower ration (20.06 ± 0.5 mm^3 and 14.65 ± 0.5 mm^3 respectively).

The weight of the protective mucus around the egg did not change in successive clutches (Table 5.1). However, the amount of capsular protection invested by the females was a function of temperature but not ration level (Table

5.1). Females maintained in warmer water laid eggs consisting of approximately 50% protective mucous, which was 10% more than those eggs laid by females in cooler water. This trend was only evident in the first clutch (t = -3.09, df = 16, p = 0.007) and not in the second clutch (t = -1.95, df = 10, p = 0.08). Although only two successive clutches were included in the ANOVA, some eggs from the third clutch deposited by females held at 11°C were unattached and had no protective mucous (Fig 5.2).

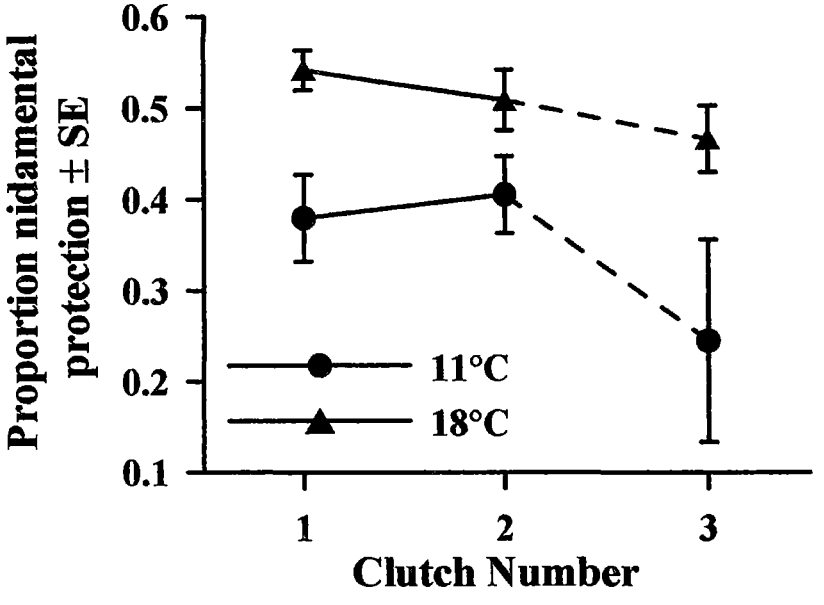


Figure 5.2. The average proportion of nidamental mucous protection (± standard error) over successive clutches for *Euprymna tasmanica* maintained at summer (18°C) and winter (11°C) temperatures. Dashed lines represent data not included in the analysis.

On average each egg mass, minus protective mucous, was 85.0 ± 0.62% water and the egg-yolk lipid content represented 6.78 ± 0.27% of the egg dry weight. There was no evidence that the lipid content changed with successive clutches (Table 5.1), but overall females fed at higher rations produced eggs with ~2% more lipid than females fed at low ration (Table 5.1). Significant differences in lipid content were only detected in the first clutch (t = -3.05, df = 16, P = 0.008) and not the second (t = -2.11, df = 10, P = 0.06) (Fig 5.3). Although the analysis did not include data from the third clutch, lipid levels dropped to <3.0% for those females (n = 2) maintained on the low ration. Five major lipid classes were identified in the eggs; wax esters (WAX), triacyglycerols (TAG) ('reserve' lipids), free fatty acids (FFA),

sterols (ST), and polar lipids (PL) ('structural' lipids). The structural polar lipids represented 77.8 ± 2.9% of the total lipid extract whereas reserve lipids were less abundant representing approximately 3.0 ± 1.0%. Free fatty acids comprised 17.5 ± 2.4%, whereas sterols and wax esters contributed 0.1 ± 0.1% and 1.5 ± 0.3% of the total lipid extract respectively. All treatments showed considerable variation in the relative quantities of these lipid classes, consequently no significant differences (split-plot ANOVAs all P values >>0.05) were detected in egg quality as a result of maternal condition (Table 5.2).

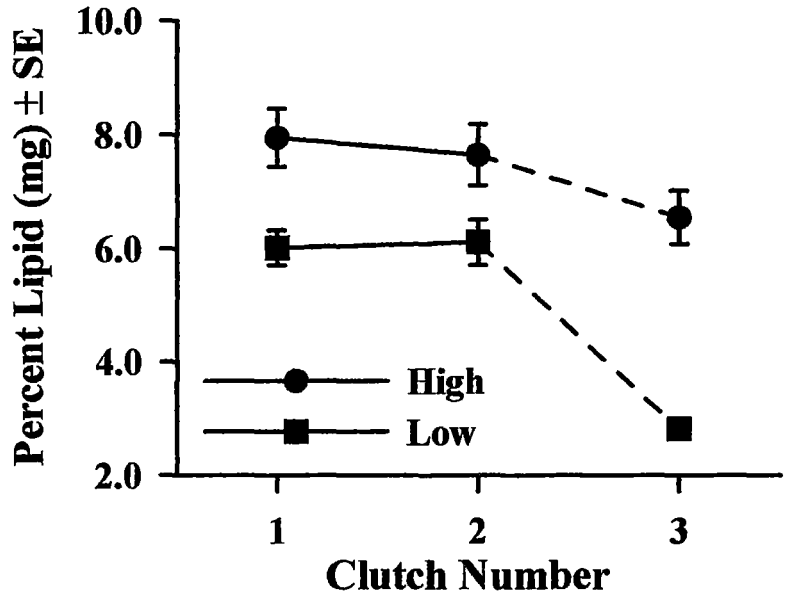


Figure 5.3. The average proportion of egg-yolk lipid (± standard error) over successive clutches for mature *Euprymna tasmanica* maintained at high and low feeding regimes. Dashed lines represent data not included in the analysis.

The fatty acid profiles of *E. tasmanica* eggs consisted of approximately 48-56% saturated, 5-14% monounsaturated, and 27-45% polyunsaturated fatty acids and were largely dominated by 16:0, 18:0, 20:5ω3 (EPA) and 22:6ω3 (DHA) (Table 5.3). No statistical difference in their relative proportions, however, were detected (split-plot ANOVAs all P values >>0.05) across treatments, suggesting that egg quality in terms of the dominating fatty acids was similar regardless of maternal condition.

Eggs incubated at 18°C developed and hatched within ~40 days, more than three times faster than those incubated at 11°C where the developmental process took approximately 135 days. The relative embryonic duration did not affect mortality

rates as no significant difference was detected between temperatures (Table 5.1). There were significantly different levels of mortality between successive clutches and between the two rations (Table 5.1). Embryo mortality was consistently higher in developing embryos deposited by low ration females, increasing from 55% dead in the first clutch to 84% dead in the second (t = -2.752, df = 12, p = 0.018). In the third clutch produced by low ration females, which was omitted in the analysis due to poor replication (n=3), 100% of the embryos failed to successfully develop with eggs exhibiting either convoluted, opaque yolk structure or grossly malformed embryos. In contrast, embryo mortality was <20% for the first two clutches in high ration females but increased 3-fold by the third clutch (Fig. 5.4). There was a strong negative correlation (Pearson's correlation r = -0.79, n = 43, P <0.01) between percentage of dead embryos and egg mass density (Fig. 5.5).

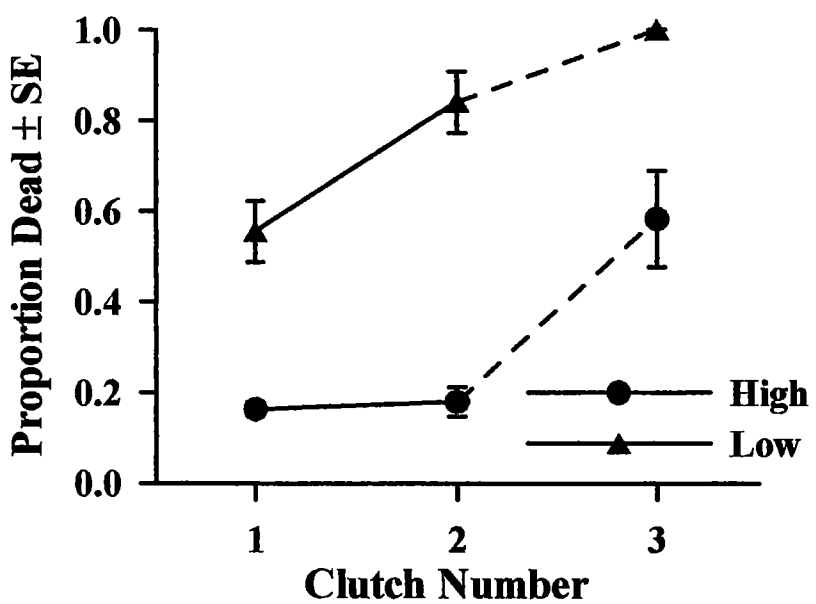


Figure 5.4. The average proportion of dead embryos (± standard error) over successive clutches for mature *Euprymna tasmanica* maintained at high and low feeding regimes. Dashed lines represent data not included in the analysis.

Hatchling length-weight relationships were very poor (r^2 = 0.12, n = 162, P <0.001) due to the spherical, globular morphology of the dumpling squid. Size of hatchlings was significantly affected by the ration level of the females (F = 16.79, df = 1, 4, P = 0.02) with high ration females producing significantly larger hatchlings than low ration females (1.73 ± 0.03, 1.20 ± 0.05 µg respectively). No correlation

between female weight and average hatchling weight was detected ($r = 0.36$, $n = 8$, $P = 0.22$).

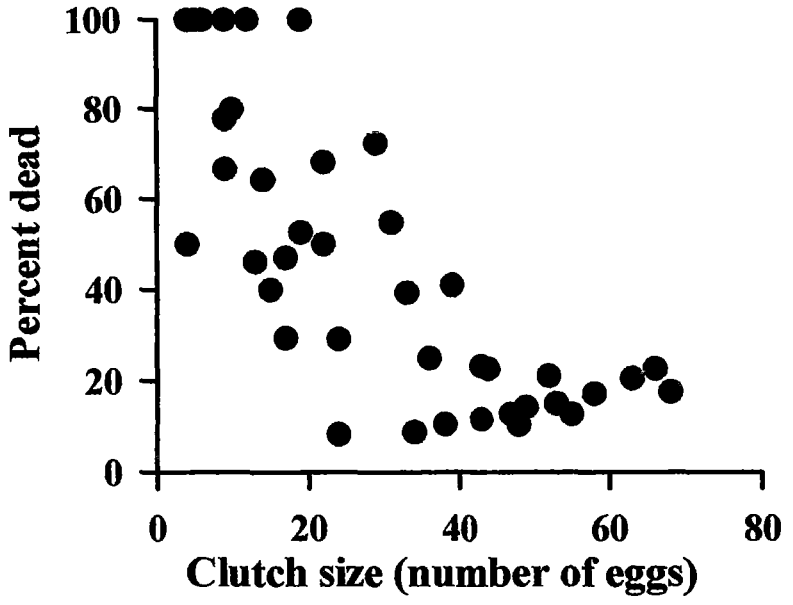


Figure 5.5. Proportion of dead *Euprymna tasmanica* embryos in relation to clutch size.

5.5. Discussion

Differences in reproductive output and embryo mortality in *Euprymna tasmanica* were largely attributed to maternal ration and not temperature. Captive females maintained on low ration consistently produced smaller clutches, consisting of smaller eggs and exhibiting higher embryo mortality rates compared to females maintained on high ration. In addition, responses in these variables were dependent on clutch sequence with both egg quantity and relative hatching success declining over successive clutches. Lipid content was significantly lower in low ration females, however the relative quality in terms of lipid class and fatty acid constituents was maintained regardless of treatment and clutch sequence. This suggests that *E. tasmanica* trades off fecundity and egg size (and as a result lipid quantity) to preserve egg quality in terms of lipid constituents. Reduction in lipid quantity may, therefore be responsible for elevated mortality rates suggesting that embryos are effectively running out of lipid reserves to fuel embryogenesis.

Like most cephalopods *E. tasmanica* undergoes direct embryonic development with juveniles hatching out as miniature versions of adults thereby avoiding an extensive larval phase (Hanlon 1990). In addition hatchlings immediately adopt a holobenthic lifestyle and are consequently not highly dispersive. The developmental process is lengthy and depending on incubation temperature can extend to approximately 4.5 months, therefore sufficient maternally derived yolk resources are needed to fuel embryonic development and ensure hatching success. It is possible that eggs spawned by low fed females have not been allocated their full complement of resources and therefore not adequately fuelling the entire developmental process, potentially accounting for increased developmental error and mortality observed in this treatment. The nutritional requirements, in terms of prey type and feeding rates for wild *E. tasmanica* are unknown and it is possible that feeding regimes enforced on captive females are nutritionally deficient potentially pushing their reproductive limits and forcing females to partition extremely limited resources.

A negative correlation between fecundity and food availability is evident in various iteroparous marine invertebrates (Rey-Rassat et al. 2002) and there are a number of theories suggesting why this trade-off occurs. These theories can be divided into two broad categories; those relating to the environment experienced by developing young and those relating to the phenotypes of the spawning females (Parker and Begon 1986). In the first category it is generally hypothesised that during periods of limited food availability small clutches are laid to reduce competitive interactions between siblings to ensure a greater proportion survive (Roff 1981). In the second category the emphasis shifts toward the survival of the mother, where it is considered advantageous for the female to direct less energy into reproduction when food is limited and therefore survive to breed again when food availability increases (Roff 1992). Either way it is analogous to a cost-benefit analysis where the main aim of the female is to maximise the success of her reproductive output and counterbalance high mortality rates (Boletzky 1988). Observing this life history strategy in *E. tasmanica* is therefore not surprising, as it is a holobenthic, shallow-water species that occupies a niche where prey availability is spatially patchy and temporally unpredictable. However, it does not explain the high incidence of embryo mortality observed in eggs produced by low ration females.

Decreasing egg volume accompanied the reduction in clutch size in low fed *E. tasmanica*. This phenomenon is not often observed, as typically marine invertebrates produce few large eggs when food is limited and more small eggs when food is abundant; trading-off high survival with high dispersal (Qian and Chia 1991). Variation in egg size and organic content can be indicative of nutritional (Bayne et al. 1975; Bayne et al., 1978) or behavioural stress (McCormick 1999) and some starved molluscs produced fewer and smaller eggs (eg *Tenellia adspersa* Chester, 1996). This pattern is not always consistent with some species maintaining egg size independent of food availability (eg Lewis and Choat 1993), however maintaining egg size does not necessarily equate to maintaining egg quality (Thompson, 1982; Cheung and Lam 1999). Although egg quality in terms of the main lipid (TAG, PL) and fatty acid constituents (EPA, DHA) was preserved regardless of treatment in *E. tasmanica* the proportionate quantity of lipid (dry weight) was significantly reduced in low fed females.

The degree to which females respond to nutritional stress may vary as a function of environmental conditions, their genetic background, or possibly their previous reproductive or nutritional history (Kristjánsson and Vøllestad 1996, Rey-Rassat et al. 2002). Reduction in batch fecundity and egg reserves through successive spawning events across all treatments in *E. tasmanica* suggests that there is a sequential draining of reproductive resources. In general cephalopods are short-lived with the majority of species exhibiting sub annual life cycles, therefore females that lay multiple clutches of eggs have to do so over a brief life span (Maxwell and Hanlon 2000). Females living in a food limited environment and possessing inherent reproductive time constraints may not be able to acquire sufficient resources in between spawning events to maintain batch fecundity. As a result female condition deteriorates and batch fecundity is compromised.

Examples of declines in batch fecundity over successive spawning episodes and subsequent hatching success in iteroparous species are rare within the literature due to the difficulties of tracking individuals in the wild and a paucity of laboratory studies. However the female calanoid copepod, *Calanus helgolandicus* maintained on low ration decreased clutch size throughout its reproductive history (Rey-Rassat

et al. 2002). Furthermore, these eggs exhibited poorer hatching success, similar to that observed for *E. tasmanica*, and it was attributed to females not able to maintain a maximum egg production rate because of the exhaustion of their metabolic reserves (Rey-Rassat et al. 2002). A field study estimating fecundity and describing processes of egg release in oceanic squids (*Illex illecebrosus, I. argentinus* and *I. coindeti*) make similar inferences, suggesting that it is likely that these species exhibit a multiple 'descending' type of spawning strategy where it is initially food driven but becomes dependent on metabolism drawn from body stores (Laptikhovsky and Nigmatullin 1993). Female condition was not measured in the present study, and future experiments assessing female condition by destructive sampling would investigate the cost of reproduction on food stressed mothers throughout multiple spawning episodes.

It was anticipated that the proportion of nidamental mucous invested by the mother to protect individual eggs would also change as a function of ration as seen in the scavenging intertidal gastropod *Nassarius festivus* (Cheung and Lam 1999). Instead no difference was found between the two feeding regimes, however significant differences were detected as a function of water temperature. Developmental rates at 11°C are considerable slower than at 18°C where embryos are encapsulated for approximately 4.5 months compared to one month. Consequently it is assumed that the longer the duration of embryogenesis the higher the need for protection to counteract embryo mortality (Boletzky 2003). However, this study indicated an opposite trend where eggs laid in warm water had 10% more capsular protection than those laid in cool water. It is possible that eggs developing in warmer water need more protection for fouling organisms and increased UV radiation. Further manipulative experimentation is required to investigate this relationship.

The effects of maternal condition will have both a direct effect on the embryo survival and an indirect flow-on effect on the competency of the resulting hatchling. Female *E. tasmanica* maintained on high ration produced larger offspring than females on low ration. Size at hatching is considered to be a relatively good indicator of hatchling competency and is of potential importance for survival (Pepin, 1989; Chambers and Trippel 1997). Larger hatchlings may have less susceptibility

to predation and starvation due to enhanced swimming abilities and predation competency in comparison to smaller hatchlings (Pepin 1989; Chapter six). Maternal nutrition may therefore have major ramifications, not only in the success of embryogenesis, but also in the subsequent traits of the hatchlings and effectively pre-determining the offspring's chances of survival.

Variation in egg quality is potentially one of the limiting factors in the successful production of offspring (Laine and Rajasilta 1999) and on the whole maternal effects on embryo mortality have been largely overlooked (Scott et al. 1999). Through this study it can be concluded that maternal ration and reproductive history plays an important role in determining batch fecundity, egg size, hatching success and potentially hatchling competency. Although extrapolating laboratory results should be treated with caution such maternal effects may have special relevance in the context of fisheries and aquaculture. For squid species that spawn multiple clutches over an extended spawning season such as, *Sepioteuthis australis* (Moltschaniwskyj et al. 2002) and *Loligo vulgaris reynaudii* (Augustyn et al. 1998), there is potential for a reduction in egg viability towards the end of the spawning season when the population lays its final clutches. Such factors that influence early mortality rates have the potential to significantly affect the magnitude of subsequent recruitment events in squid populations.

Table 5.1. Summary of the split-plot repeated measures ANOVA output comparing batch fecundity, proportion mucus protection, rates of embryo mortality and lipid levels with ration level and temperature and across successive clutches.

	Clutch Size (ln eggs)			**Nidamental Mucous**			**Embryo Mortality**			**Lipid Content**		
Number of clutches		**3**			**2**			**2**			**2**	
Sources of variation	***F***	***df***	***p***	***F***	***df***	***p***	***F***	***df***	***p***	***F***	***df***	***p***
Between Subjects												
Temperature	0.37	1	0.55	9.42	1	**0.02**	2.28	1	0.16	0.97	1	0.35
Ration	12.95	1	**<0.01**	4.03	1	0.08	75.99	1	**<0.01**	15.95	1	**<0.01**
Temp*Ration	0.97	1	0.34	1.01	1	0.35	1.71	1	0.22	3.28	1	0.11
Within Subjects*												
Clutch	16.58	2	**<0.01**	0.01	1	0.91	10.87	1	**<0.01**	0.36	1	0.57
Temp*Clutch	0.46	2	0.62	0.63	1	0.45	<0.01	1	0.99	0.27	1	0.62
Ration*Clutch	0.52	2	0.59	0.01	1	0.93	7.40	1	**0.02**	0.38	1	0.56
Temp*Ration*Clutch	1.45	2	0.25	0.54	1	0.49	<0.01	1	0.95	0.09	1	0.77

Table 5.2. Relative proportions of lipid classes (mean ± sd) over each treatment combination.

Temp	11	11	11	11	11	11	18	18	18	18	18	18
Ration	**Low**	**Low**	**Low**	**High**	**High**	**High**	**Low**	**Low**	**Low**	**High**	**High**	**High**
Clutch	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
WAX	0.2 ± 0.3	0.7 ± 0.3	0.5 ± 0.2	1.5 ± 0.6	2.3 ± 1.1	1.6	2.2 ± 1.2	2.0 ± 3.3	.	1.6 ± 1.0	1.9 ± 0.6	1.7 ± 0.7
TAG	2.9 ± 1.5	2.4 ± 0.5	5.9 ± 3.4	1.6 ± 1.6	1.0 ± 0.8	1.4	2.4 ± 2.3	0.6 ± 0.9	.	3.2 ± 3.6	4.7 ± 2.1	4.5 ± 5.1
FFA	19.9 ± 17.8	23.0 ± 9.7	10.8 ± 11.8	15.6 ± 3.0	12.2 ± 9.3	14.1	15.4 ± 3.8	10.8 ± 12.6	.	16.4 ± 4.5	19.8 ± 3.3	21.4 ± 5.3
ST	0	0	0	0.1 ± 0.2	0	0	0.4 ± 0.7	0	.	0.2 ± 0.4	0	0.2 ± 0.3
PL	76.9 ± 18.7	73.8 ± 10.8	82.8 ± 8.7	81.2 ± 1.4	84.5 ± 9.7	82.9	79.5 ± 4.9	86.6 ± 16.8	.	70.3 ± 16.6	73.6 ± 3.4	72.3 ± 10.4

Table 5.3. Relative proportions of fatty acid constituents over each treatment combination. Standard deviations = 10 – 15% of the mean.

Temp	**11**	**11**	**11**	**11**	**11**	**11**	**18**	**18**	**18**	**18**	**18**	**18**
Ration	**Low**	**Low**	**Low**	**High**	**High**	**High**	**Low**	**Low**	**Low**	**High**	**High**	**High**
Clutch	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
SATURATES												
14:0	2.03	1.05	1.8	1.51	1.40	1.4	0.77	0.64	-	0.71	0.67	0.76
15:0	0.06	0.12	0.1	0.14	0.10	-	0.10	-	-	0.10	0.09	0.13
16:0	34.58	34.60	37.6	31.39	32.59	33.8	31.97	29.31	-	31.10	30.04	31.42
17:0	1.21	0.61	1.6	1.40	1.47	1.4	1.62	7.97	-	1.51	1.49	1.42
18:0	17.77	16.66	17.6	15.22	16.84	15.9	16.84	17.61	-	15.68	16.47	16.31
20:0	0.06	0.12	<0.1	<0.1	<0.1	-	-	-	-	0.33	-	-
i15:0	-	-	-	-	-	-	-	-	-	<0.1	-	-
i17:0	-	-	-	<0.1	-	-	-	-	-	<0.1	<0.1	-
i18:0	-	-	-	-	-	-	-	-	-	<0.1	<0.1	-
MONOUNSATURATES												
16:1	0.20	0.19	-	0.22	-	0.4	1.02	-	-	-	0.21	0.43
16:1ω7	0.24	0.29	-	0.27	-	-	0.28	-	-	0.77	0.22	0.40
17:1	-	-	-	-	-	-	-	-	-	-	-	0.03
18:1ω9	3.20	2.08	4.0	1.24	0.10	1.0	1.82	1.38	-	0.62	0.65	0.89
18:1ω7	5.01	4.04	5.7	3.82	2.86	5.4	2.35	4.15	-	3.00	2.76	4.63
20:1ω9	1.77	1.21	2.7	2.12	2.06	2.0	2.39	2.20	-	1.33	2.15	2.09

Table 5.3. Continued...

Temp	**11**	**11**	**11**	**11**	**11**	**11**	**18**	**18**	**18**	**18**	**18**	**18**
Ration	**Low**	**Low**	**Low**	**High**	**High**	**High**	**Low**	**Low**	**Low**	**High**	**High**	**High**
Clutch	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
POLYUNSATURATES												
18:2ω6	0.01	0.42	7.3	2.12	5.61	-	0.24	-	-	0.20	-	-
18:3ω6	-	-	-	0.21	-	-	0.17	-	-	0.08	1.20	0.28
18:3ω3	-	-	-	-	-	-	-		-	1.85	-	-
18:4	-	0.41	-	0.20	-	-	0.26	-	-	0.16	1.36	0.07
20:2ω6	0.02	2.32	-	-	-	-	-	-	-	-	-	-
20:4ω6	0.02	0.30	-	0.22	0.05	-	-	-	-	0.76	0.37	0.09
20:4ω3	-	0.25	-	0.19	-	-	0.25	-	-	0.10	0.26	0.28
20:5ω3	17.29	19.16	12.4	20.75	21.08	18.0	19.05	16.85	-	22.48	21.80	21.89
22:5ω3	1.06	0.50	-	0.92	0.50	0.8	0.69	0.40	-	0.60	1.13	0.15
22:6ω3	15.64	18.71	15.0	18.72	18.24	20.0	20.37	19.48	-	18.96	19.42	19.60
SUMMARY												
Total	100.17	103.03	105.7	100.7	102.9	100.0	100.2	100.0	-	100.3	100.3	100.9
PUFA ω3	33.99	38.62	27.32	40.60	39.82	38.78	40.35	36.73	-	43.98	42.61	41.93
PUFA ω6	0.05	3.03	7.27	2.55	5.67	0.00	0.42	0.00	-	1.04	1.56	0.37
DHA/EPA	0.90	0.98	1.2110	0.9022	0.8651	1.1103	1.0693	1.1558	-	0.8436	0.8906	0.8953

CHAPTER SIX

ARE BIGGER CALAMARY *SEPIOTEUTHIS AUSTRALIS* HATCHLINGS MORE LIKELY TO SURVIVE? A STUDY BASED ON STATOLITH DIMENSIONS.

Steer, MA, GT Pecl, NA Moltschaniwskyj (2003) Marine Ecology Progress Series 261: 175-182

6.1. ABSTRACT

To determine if any size selective processes were operating throughout the squids' life history this study set out to explore whether bigger hatchlings are more likely to survive to adulthood. This was achieved by comparing natal statolith dimensions between recently hatched (< 13 hrs old) and successfully recruited adult *Sepioteuthis australis*. The squid statolith (analogous to the teleost otolith) retains a check associated with hatching, and the natal radius (NR) at hatching had a strong linear relationship to dorsal mantle length (ML). Hatchlings were collected from October (2001) to February (2002) on natural spawning grounds located on the east coast of Tasmania using emergent traps. Hatchling size was extremely variable ranging from 4.3 to 7.3 mm (ML), with significantly larger animals hatching out in November and the smallest in February. From February to August adults were collected from the same bay and aged using validated daily rings in the statolith, those adults which were estimated to have been born between October and February were included in the analysis. In all but one month a significant difference between the NR size distributions of the hatchlings and adults was detected due to low numbers of adults with small natal radii. This indicated that smaller hatchlings were less likely to recruit, suggesting that there is an element of size-mediated mortality operating on populations of *S. australis*.

6.2. INTRODUCTION

Given the short life span (< one year) and low fecundity of neritic squid high survival rates during the early life history are essential to guarantee that recruitment failure does not occur. However, currently there are no estimates of juvenile mortality rates for neritic squid and it is not clear if size at hatching is important in survivorship. It is widely suggested in the fish literature that fast growing and rapidly developing larvae attain larger sizes earlier and therefore lower the risk of size-dependent mortality (Johannessen et al. 2000). There are a number of theories supporting why this is the case and they collectively attribute it to faster growing fish accelerating through the window of vulnerability associated with being small and

poorly developed (Fogarty et al. 1991). Furthermore, this potential for faster growth may be present at hatching, with larger hatchlings displaying an 'athletic' edge compared to their smaller siblings (Meekan and Fortier 1996). Similarly some individuals may be more likely to survive based on other physical, behavioural, or physiological characteristics (Rice et al. 1993).

Studies exploring size-selective mortality in teleost fish have used otolith dimensions to estimate the length of an individual. This is achieved by generating a predictive relationship between otolith size and fish size, and allowing the size of that fish at an earlier stage of its life history to be estimated (Francis 1990). This approach, however, may fail if there is an uncoupling between somatic and otolith growth (Molony and Choat 1990; Mosegaard 1990). Establishing a link between otolith size and larval size at hatching may be more appropriate in addressing size selective processes, although difficult in wild fisheries due to poor availability of recently hatched larvae. Weak positive correlations between otolith size and hatchling size for laboratory reared Atlantic cod *Gadus morhua* (Miller et al. 1999) and wild-caught juvenile Sockeye Salmon *Oncorhynchus nerka* (West and Larkin 1987), do not provide reliable predictions to estimate hatchling size from adult otoliths. However, constraining the data so that it is stock- and season-specific may potentially account for some of the variability and improve the predictive power of this approach (Miller et al. 1999).

In squid populations establishing and identifying characteristics of successful recruits in non-overlapping generations may lead to improved forecasts of each year's population strength. This is particularly pertinent in the face of increasing worldwide cephalopod fishing pressure (Roper and Rathjen 1991). The statoliths in squid are structurally and functionally analogous to fish otoliths and as a result many of the ageing and growth techniques used in fish otolith analysis are applicable in squid (Jackson 1994). Linear relationships (of varying strengths) between statolith dimensions and body size and weight at hatching are evident for some laboratory reared cephalopods (ie *Sepioteuthis lessoniana*, Ikeda et al. 1999; Jackson and Moltschaniwskyj 2001; *Loligo vulgaris*, *Loligo forbesi*, Martins 1997). Loliginid squid generally display a distinct anomaly, 'hatch-check' or 'natal ring', in the statolith structure, associated with the day of hatching (Villanueva et al. 2003). The

retention of this anomaly in the adult statolith potentially provides a way of estimating an adults size at hatching from the size of the statolith radii at hatching (Ikeda et al. 1999). This method potentially provides a powerful tool for exploring the 'bigger is better' hypothesis by collecting hatchlings over the spring/summer spawning period and adults three to four months later and comparing the size frequency of the hatch check radii.

The primary aim of this study was to investigate if size selective mortality was operating during *Sepioteuthis australis'* life history. If no size selection is operating during the squids' life history, then the size frequency of the natal statoliths in recent hatchlings and those preserved in adults would be similar. If, however, size selective mortality has occurred with bigger hatchlings more likely to survive, then the natal radii size distribution of the adult statolith would be expected to be further to the right of the hatchling natal radii size distribution. The advantage of this approach is that it is field-based and avoids any biases associated with the potential uncoupling of somatic and statolith growth. To reduce the variability associated with stock- and season- specific affects, we limited collection of hatchlings and adults to specific areas and times of the year.

6.3. MATERIALS AND METHODS

6.3.1. Validation of hatchling statolith and somatic relationships

Recent (<13 hrs old) *Sepioteuthis australis* hatchlings were collected from inshore spawning beds located within Great Oyster Bay, Tasmania (Fig 6.1) from late October 2001 to early February 2002, excluding January due to poor weather. Great Oyster Bay represents a unique area as it accommodates the majority of *S. australis* spawning activity on the east coast of Tasmania, comprising 50-70% of Tasmania's commercial catch (Lyle and Hodgson 2001). As a result it was assumed that squid hatching out in Great Oyster Bay were highly likely to use the same area to spawn as adults.

Hatchlings were captured using purpose built emergent traps, which were placed and anchored over egg masses from which individuals were hatching (Figure

6.2). Traps were constructed from 1.5 mm mesh and <1.0 mm perforated plastic collection vials that retained all hatchlings. Five emergent traps were deployed on shallow (<4m) productive spawning beds in Great Oyster Bay within 10 km of each other (Fig 6.1). Each trap was inspected twice a day (at ~0800 and ~1900 hrs) over four consecutive days in each month. Captured hatchlings were taken ashore and immediately preserved in 70% ethanol. Each hatchling was weighed (g) and dorsal mantle length (ML) measured (mm) using a stereo dissector and eyepiece graticule. Statoliths were removed from the hatchlings by decapitating the squid and teasing each statolith from the exposed statocyst chamber. Statoliths were rinsed in 100% ethanol to remove excess tissue, air-dried and whole mounted on the posterior plane in Crystal Bond© thermoplastic cement.

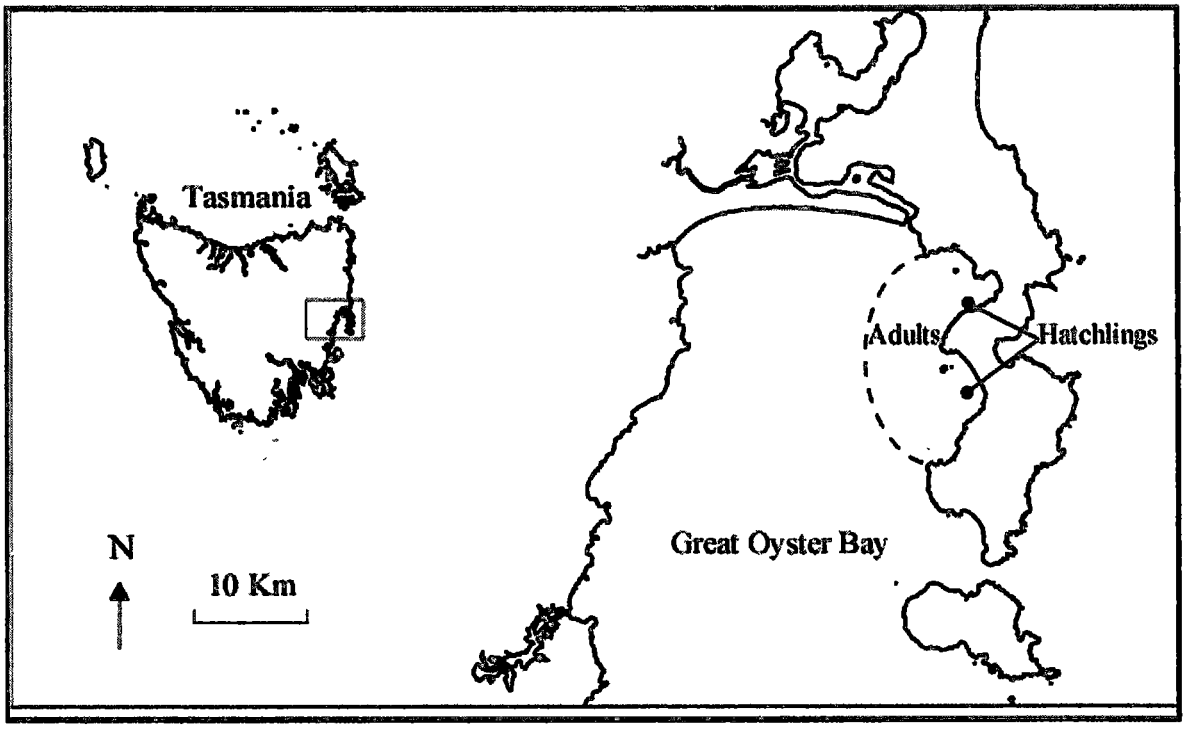


Figure 6.1. Map of Great Oyster Bay, located on the east coast of Tasmania, indicating where hatchlings and adults were collected for this study.

Natal statolith total length (TL) and radius (NR), measured from the nucleus to the statolith margin perpendicular to the longitudinal axis (Fig 6.3) were measured using a high power binocular microscope and Scion image analysis computer program. These dimensions were chosen because these natal ring dimensions can be reliably measured in prepared adult statoliths, depending on whether they have been ground transversely or dorso-ventrally. To determine whether there was a difference in dimensions between the left and right statoliths, both statoliths from 50 random hatchlings were measured and compared using paired *t*-tests. No difference was detected for either TL (t = 0.56, df = 49, P = 0.58) or NR (t = -0.15, df = 49, P = 0.88), therefore only one statolith was used from the remaining hatchlings. To determine the reliability of the measured linear dimensions a second person measured TL and NR in 20–25 statoliths. No reader bias was evident for either linear dimensions (TL, t = 1.72, df = 24, P = 0.12 and NR, t = 1.73, df = 19, P = 0.09).

6.3.2. Measuring the 'natal radius' in adults

Adults were caught in Great Oyster Bay (within a 10 km radius from where hatchlings were collected) from February and August. Unfortunately in 2002 low numbers of adults were caught (n = 24), therefore adults that had been caught in 1996 to 2001 were used. For the purposes of this study it was assumed that year-to-year variation was minimal, but will require further investigation. Each individual was processed fresh; sexed, weighed, measured (ML) and both statoliths were extracted, rinsed in 100% ethanol and stored dry. Grinding an adult statolith down to the focus on the lateral plane allows TL and NR to be measured. However, the natal statolith's rostrum is occasionally obscured by the growth of the wing preventing measurement of TL. Grinding to the focus dorso-ventrally was adopted in this study due to the precision of measuring NR. Statoliths were whole mounted in Crystal Bond© thermoplastic cement with the ventral dorsal dome projecting over the edge of the glass slide. The statolith was then ground along a transverse plane, using wet 1200 μm carborundum paper, until the plane passed through the statolith nucleus. The ground surface was polished with 0.05 μm alumina powder on wet suede polishing cloth. The extent and intensity of grinding was continually monitored using a binocular light microscope (40x). The polished surface was mounted so the

rostrum was aligned perpendicular to the slide's surface. The statolith was ground and polished to a section thin enough for examination. Statoliths were heat treated on a hot plate for 1-2 minutes to accentuate the natal ring and increments. Age estimates were determined from daily increments in the statolith, validated for this species by Pecl (2000). Adults that were estimated to have been born from October to February were used in the analysis. From the dorso-ventral plane the natal ring, representing the boundary of the hatchling statolith, and the statolith nucleus is evident. The natal statolith radius (NR), measured from the nucleus to the maximum width of the natal ring was measured using Scion image analysis.

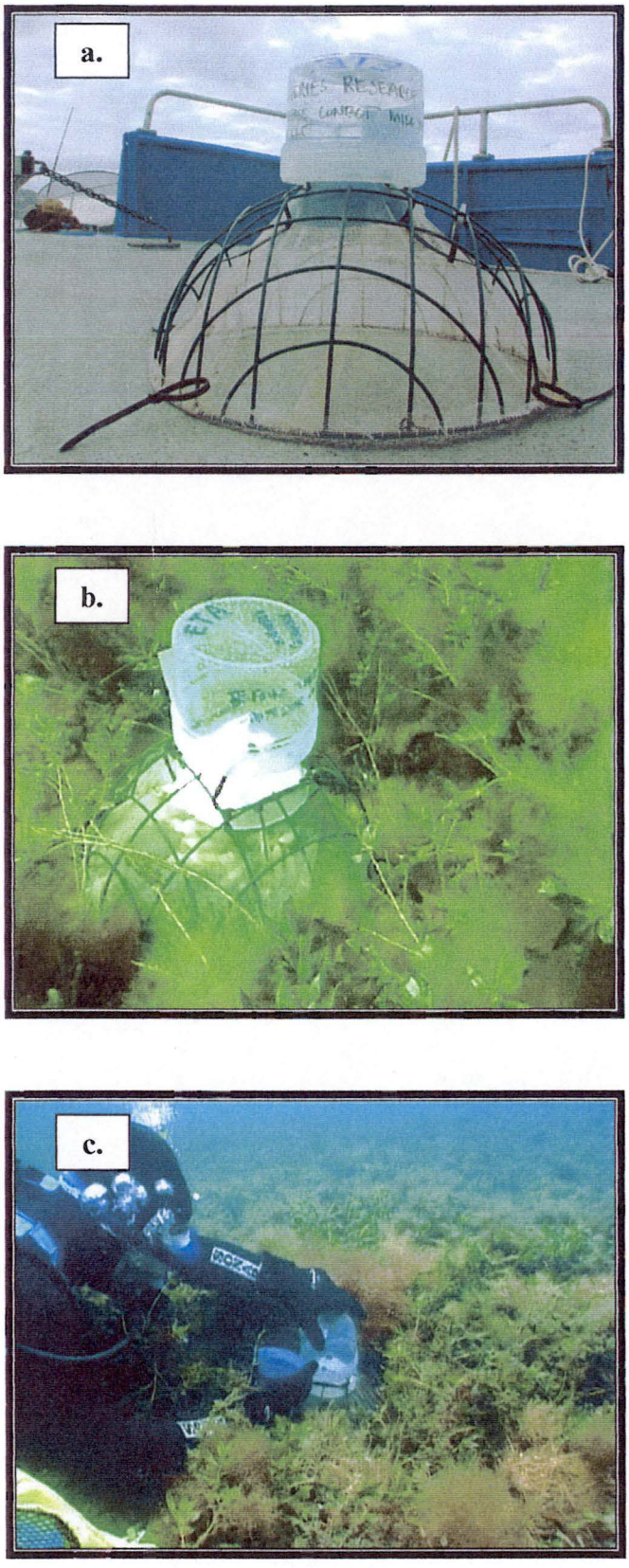


Figure 6.2. (a.) Emergent trap used to collect *Sepioteuthis australis* hatchlings. (b.) Deployed over a hatching egg mass. (c.) Collection vials able to be serviced *in situ.*

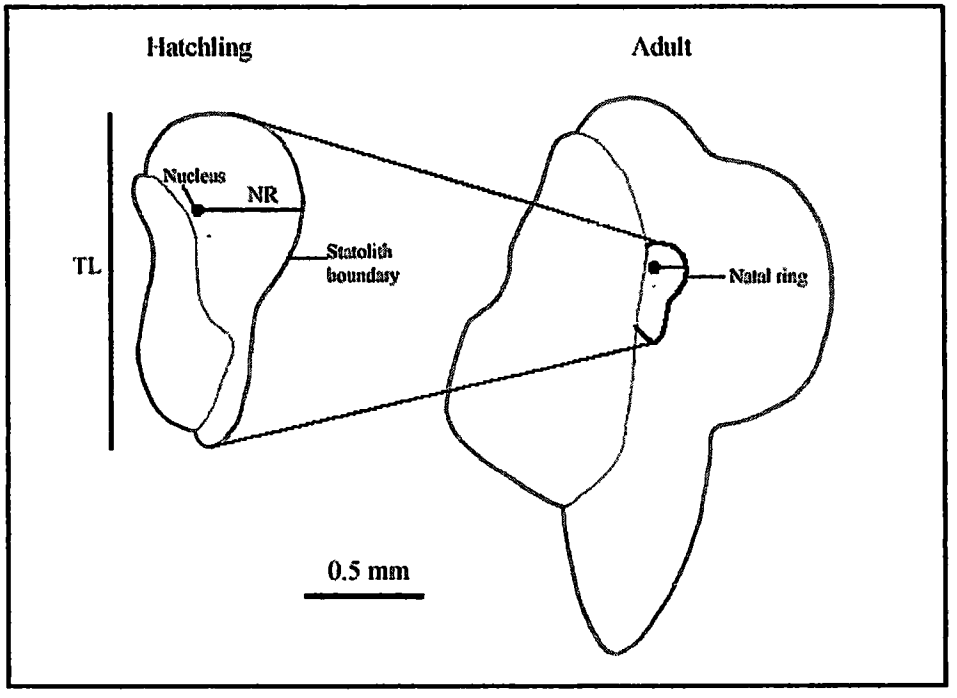


Figure 6.3. Schematic illustration of the hatchling and adult statolith, highlighting the preservation of the natal statolith in the adult form. Natal radius (NR) and total length (TL) are indicated on the hatchling statolith.

6.4. RESULTS

6.4.1. Hatchlings

A total of 364 *Sepioteuthis australis* hatchlings were collected from the Great Oyster Bay region from October through to February. Overall, hatchling ML ranged from 4.33 to 7.33 mm with significant differences in size among months ($F = 82.24$, $df = 3, 360$ $P < 0.001$). November hatchlings were as much as 4-14% larger than hatchlings in any other month, with the smallest hatchlings in February (Fig 6.4). The hatchling length-weight relationships were not significantly difference among the months (ANCOVA, $F = 1.50$, $df = 3, 356$, $P = 0.21$). Indicating that the rate of increase in weight with length was similar among months (Fig 6.5). A large proportion of hatchlings (≈60%) collected in October were observed to have retained their external yolk sac, suggesting they had been stimulated to hatch prematurely by prevailing weather conditions. Taking these pre-mature October hatchlings into

consideration it was apparent that hatchling size decreased with increasing seasonal temperatures.

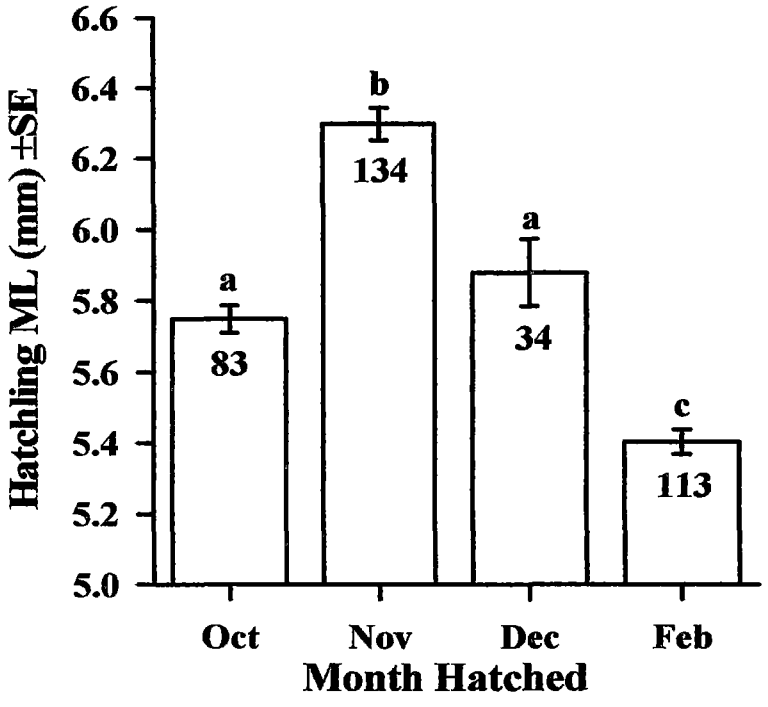


Figure 6.4. Mean hatchling mantle length (ML) ± standard error over the sampling period. Lower case letters indicate significant difference amongst means via a *post hoc* Tukey's test. Numbers indicate sample size.

At least one statolith was successfully dissected from 348 (95.1%) individuals and SL measured. In some cases (10.9%) the statolith rostrum either cracked or crumbled during the dissection process, consequently TL was measured from 310 individuals. Both measured statolith dimensions (NR and TL) were significantly and positively related to hatchling length and weight (Fig 6.6). In general larger hatchlings had larger statoliths, however, the two statolith dimensions varied in their predictive power. Hatchling natal radius (NR) was the strongest predictor of ML explaining 68% (n = 348) of the variation in ML, while total length (TL) explained slightly less (63%) of the variation in ML (n = 310). A similar trend was observed for hatchling weight, however, the predictive power was weaker explaining 53% and 45% of the variation respectively.

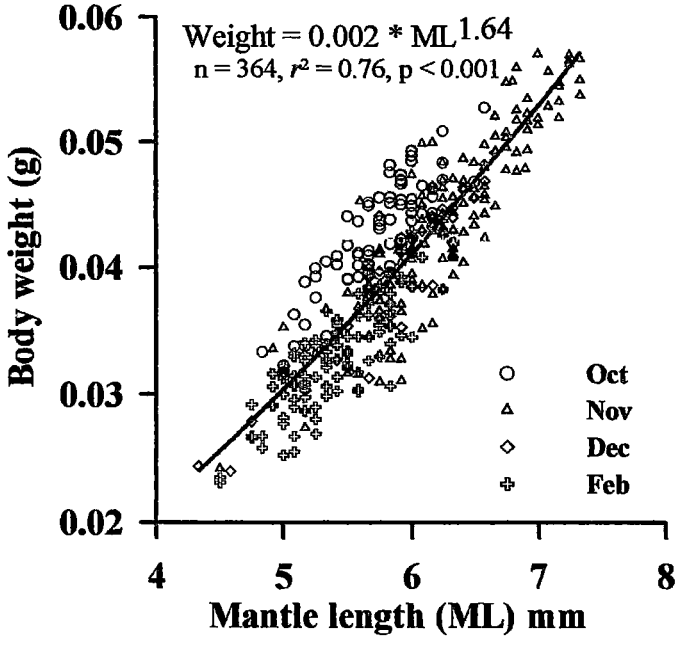


Figure 6.5. Hatchling length weight relationship. Power regression formula provided for total sample. Data points have been coded to represent sampling month.

6.4.2. Comparison of successful recruits and recent hatchlings

A total of 269 *Sepioteuthis australis* adults were successfully aged and subsequently calculated to have hatched in the target months (Oct – Feb, except Jan). There were significant differences between natal radii size frequency distributions between hatchlings and adults in each birth month, except in November (Fig. 6.7). When significant differences were detected there were proportionally more adults with natal radii in the larger size classes, resulting in distributions that were shifted to the larger end of the size spectrum. In total, 42.53% of all the collected hatchlings had natal radii >0.12 mm compared with 73.13% of the adults, demonstrating the differences in the size distributions.

No significant difference was detected in the adult natal radius size distributions among the months (mean = 0.125 mm) (Table 6.1). Using the validated ML/NR regression equation a hatchling with an average NR of 0.125 mm would have a predicted mantle length of 6.19 ± 0.05 (95% CL), which was 5.8% larger than the average size of the trapped hatchlings (5.85 ± 0.06).

A series of Pearson's correlations between adult biological parameters and predicted hatchling size were used to identify any biological characteristics of adults that would explain their survival. Correlations between predicted hatchling size with adult ML (mm), adult weight (g) and calculated residuals from the adult length-weight relationship were all weak and non significant (ML; $r = 0.12$, n = 239, P = 0.06, Weight; $r = 0.09$, n = 239, P = 0.17, Resids; $r = -0.08$, n = 243, P = 0.22). Similarly no difference was detected when comparing the predicted hatchling size distributions between sexes (Z = 0.74, n = 210, P = 0.65) suggesting that no sexual dimorphism is evident at hatching.

Table 6.1. Pairwise comparisons of the size frequency distributions of NR measurements in adult statoliths between months via a series of Kolmogorov-Smirnov (Z) tests

Comparison	n	Z	Sig
October v November	105	1.013	0.256
October v December	143	1.149	0.143
October v February	134	0.623	0.832
November v December	134	0.506	0.960
November v February	125	0.924	0.361
December v February	163	1.027	0.242

6.5. DISCUSSION

In general larger *Sepioteuthis australis* hatchlings had larger statoliths, and there was a positive relationship between natal statolith radius (NR) and mantle length (ML). The natal ring and nucleus preserved in adult statoliths were clear enough to measure NR. In three of the four months the adult natal radii were larger than the hatchling natal radii. Two major assumptions underlie this study, the first related to the formation of the natal-ring at hatching an assumption that is supported throughout the literature (Natsukari and Komine 1992; Villanueva et al. 2003) and has been validated for *S. australis* through the rearing of known age animals in captivity (Pecl 2000). The second assumption made was that there was minimal annual variation in hatchling size, which allowed us to compare adults hatched

during 1996-2001, with hatchlings from 2001. However, there was an absence of adults with small natal radii, inferring smaller sizes at hatching, in the distribution. This suggests a degree of size-selective mortality, or non-random predation, was operating throughout the early life history. Interestingly, no significant difference was detected in the predicted size at hatching across months. Most of the adults were estimated to have a hatched ML > 6.19 mm, with hatchlings smaller than this poorly represented. Although it is impossible to attribute the loss of these individuals directly to size-selective predation from the data obtained, these results suggest that the smallest hatchlings failed to recruit into the fishery.

Sepioteuthis australis hatchlings are the largest of the loliginids having relatively advanced behavioural and functional attributes (Steer et al. 2003; Chapter two). Despite this they are still susceptible to a suite of predators throughout their entire life and as a result are considered an important link in the marine ecosystem (Gales et al. 1994). Size-selective mortality is observed in a variety of marine species that exhibit broad variability in size and is often referred to as the 'bigger is better' hypothesis, where average mortality rates decrease with age and body size as the individuals' sensitivity to starvation decreases and its foraging success and swimming abilities improve (Conover and Schultz 1997). However there is potential for individuals to grow out of one window of vulnerability for a particular type of predator and enter into another suggesting that there may be continuous non-random predation, with animals always running the gauntlet. A purely size-based predation model is considered an oversimplification of the inter-connected processes involved in the marine ecosystem as there are naturally many other contributing factors (Cowan and Shaw 2002). Local hydrodynamics, availability of food, intolerance to extreme environmental conditions, susceptibility to disease/parasites, and fishing pressure may also be responsible for elevated mortality (Sogard 1997) with some of these continuing to operate with an element of size-selectivity.

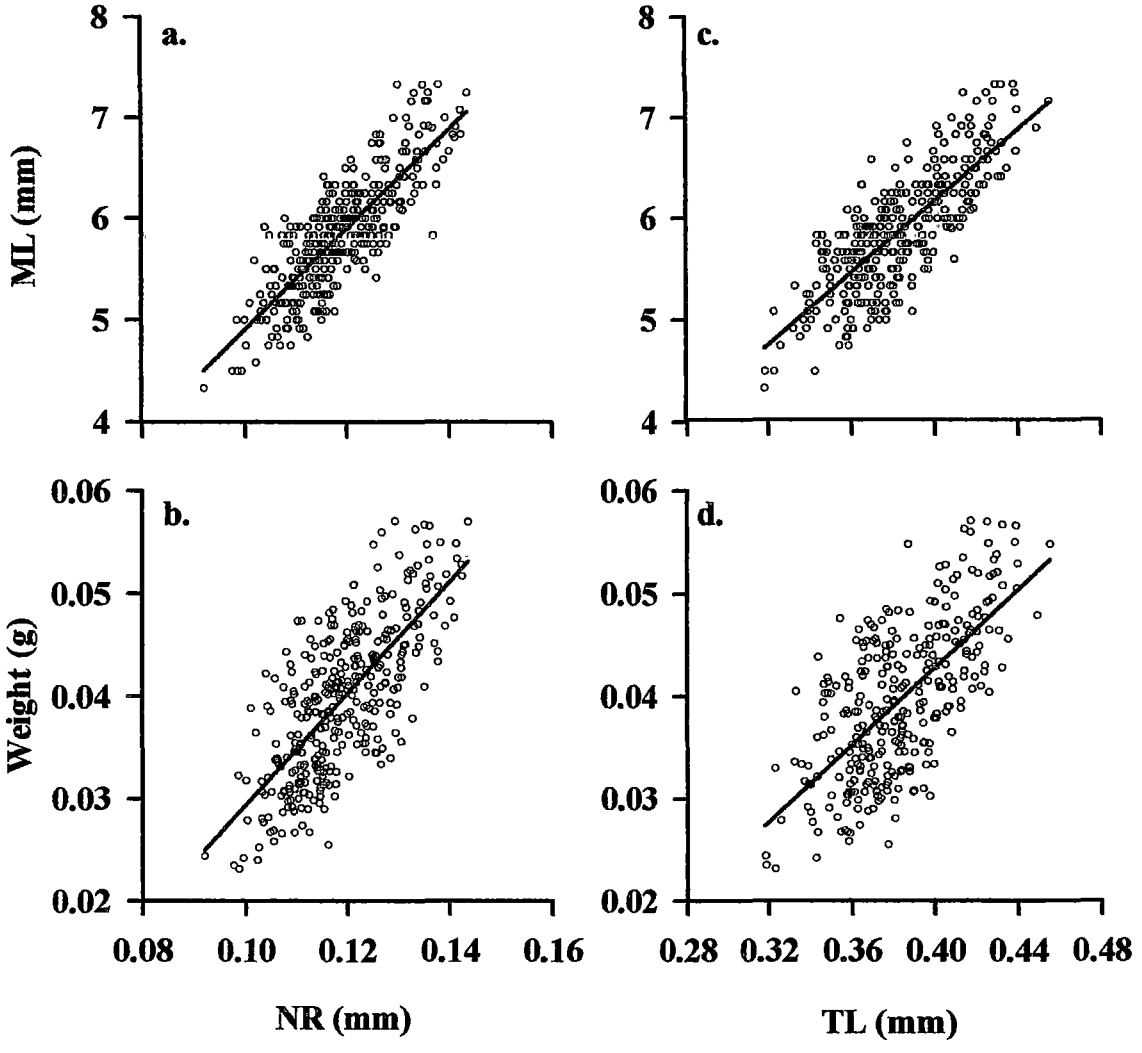
Due to the large proportion (approx. 60%) of pre-mature hatchlings collected in the October sample differences in the size distributions may be exaggerated. Size-selective mortality was not strongly evident during November, coinciding with the beginning of the 'hatching' season. However, there was a progressive shift in the frequency distributions between hatchlings and successful recruits in subsequent

months. West and Larkin (1987) observed similar trends for salmon *Oncorhynchus nerka*, with mortality increasing in intensity through late summer to early autumn and was attributed to size-mediated predation and parasitism. However, the correlation between fish and otolith length was considered too weak ($r^2 < 0.2$) to attribute losses purely to size selective mortality and it was suggested that other parameters such as weight and age be incorporated in the model to reduce biases (Mosegaard 1990). The relationship between statolith radius and hatchling length in this study was comparatively stronger ($r^2 = 0.68$) and although the susceptibility of juveniles to parasites was not investigated, exaggerated differences in the size compositions may be in part attributed to density-dependent effects. Large variation in hatchling sizes existed over the extended hatching period (also see Villanueva et al. 2003), therefore larger, older, and more competent individuals have the capacity to cannibalise their smaller and younger conspecifics, a phenomenon evident in haddock, (*Melanogrammus aeglefinus*) and bluefin tuna (*Thunnus maccoyii*) populations (Perry and Nielson 1988; Young and Davies 1990). Cephalopods are cannibalistic throughout most life stages, especially when food is limited, and there is evidence of intra-cohort cannibalism in *Sepioteuthis* hatchlings, where larger hatchlings readily attack and consume smaller conspecifics, both in the wild (Pecl, unpublished data) and in captivity (Walsh et al. 2002). Given that calamary spawn over a protracted season, the risk of mortality for hatchlings due to cannibalism may be heightened as the season progresses and egg density increases (see Moltschaniwskyj and Pecl 2003), potentially accounting for the increasing differences observed in this study. Alternatively, differences may be attributed to temporal shifts in productivity and available prey, directly challenging hatchlings upon their switch from an endogenous to exogenous mode of feeding (Cushing 1975).

Complimenting the data from this research with pre-recruit studies will determine whether natural mortality is greatest during the early life stages (Type III mortality curve, Pearl and Miner 1935) or whether predation occurs at a continuous rate throughout their entire life history (Type II). Pre-recruit, larval abundance studies on the oceanic ommastrephid *Todarodes pacificus* indicated that this species exhibited a Type III mortality curve where rhynchoteuthion larvae measuring ≤ 6 mm ML suffered higher mortality than larvae > 6 mm (Okutani and Watanabe 1983).

This shift is suggested to correspond with the end of the rhynchoteuthion stage and a period when diel migration is critical to the larvae. *Sepioteuthis australis* possess two maternally derived yolk sacs, the external yolk sac is typically depleted pre-hatching, however the internal yolk sac sustains the hatchling for a short period post-hatch (Steer et al. 2003, Chapter two). Although calamary do not undergo metamorphosis the switch in feeding modes may represent a similar critical period accounting for improved survival rates post 6.19 mm.

It has been suggested that in a population where size differences are maintained and juvenile size is a good predictor of adult size, selective fishing mortality removing relatively large individuals could obscure the interpretation of the data (Sogard 1997). Other than size at hatching there was no strong evidence of other biological parameters present in the adults that provided an indication of their 'athletic' edge. The adult population was relatively heterogeneous with all explored correlates with hatchling size yielding insignificant results. The ability of squid to quickly respond to their immediate environment in terms of growth and reproduction (Hatfield 2000; Jackson and Moltschaniwskyj 2002) may potentially cloud any functional correlations with hatchling size. Furthermore, a comparison of purse-seine (non selective) and jigging (potentially selective) sampling techniques yielded no significant difference in the size composition of captured squid (Moltschaniwskyj et al. 2003) suggesting that there were no associated collection biases in this study and the results obtained truly reflect size-dependent mortality. A concern associated with the interpretation of the data, however, relates to the amount of variability explained by the predictive regression model. Although on the whole the model is not poor, a degree of error was assumed to be incorporated as a function of measuring very small hatchlings and statolith dimensions. It has been suggested that statolith area may potentially improve the model by reducing the amount of error associated with measuring linear dimensions (Meekan et al. 1998). The methodology involved in measuring the area of the natal statolith preserved in adults has not yet been refined but is theoretically possible when grinding the statolith laterally. Although the exact processes involved in size selective mortality in southern calamary are unclear such estimates provide valuable insights into the dynamic processes and the vulnerability of small squid which can potentially be used as a proxy to predict recruitment strength.



Graph	n	Equation	r^2	P
A	348	ML = 49.59 (± 1.8) SR – 0.006 (± 0.22)	0.68	<0.001
B	348	Wt = 0.55 (± 0.03) SR – 0.025 (± 0.003)	0.53	<0.001
C	310	ML = 17.71 (± 0.78) TL – 0.91 (± 0.30)	0.63	<0.001
D	310	Wt = 0.19 (± 0.12) TL – 0.03 (± 0.005)	0.45	<0.001

Figure 6.6. Linear regressions between statolith dimensions, total length (TL) and statolith radius (NR) with hatchling mantle length (ML) and weight. Regression equations are provided to indicate each comparison's relative strength.

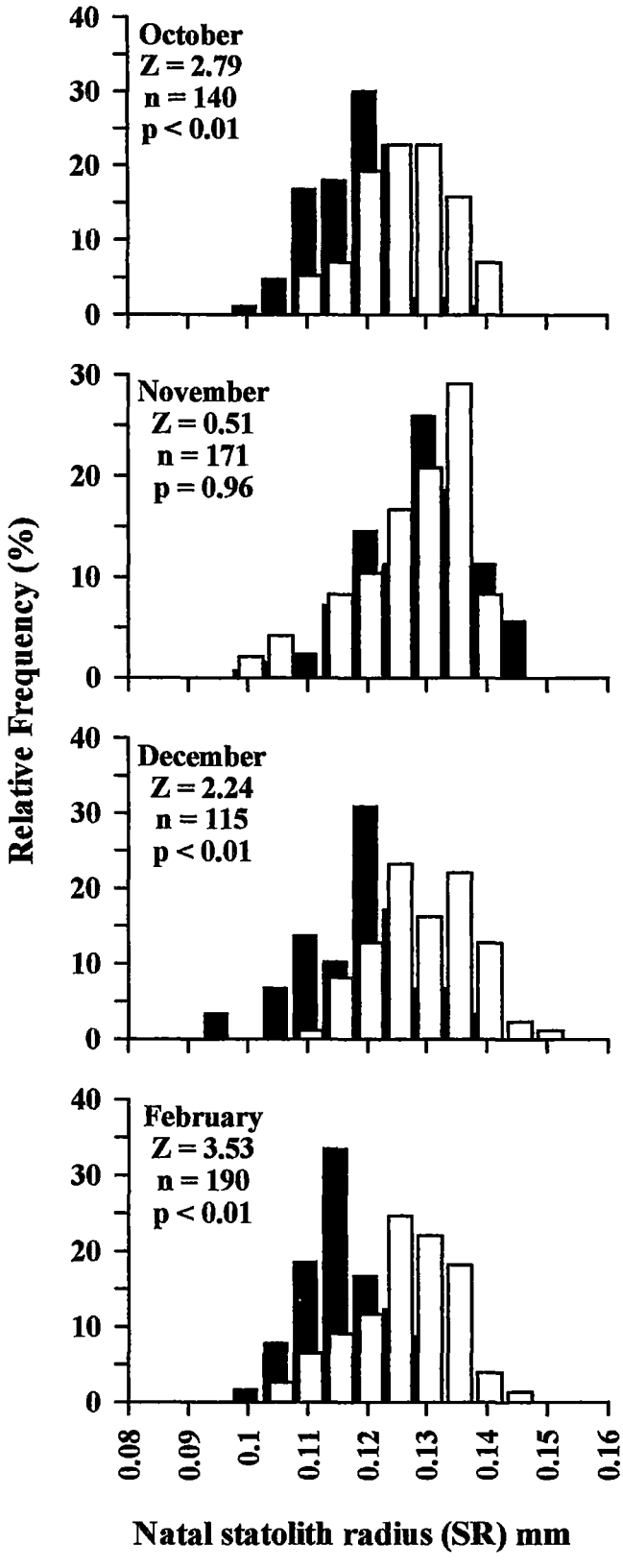


Figure 6.7. Monthly natal statolith radius (NR) length frequency histograms plotted for wild-caught hatchlings (black bars) and adults (white bars). Each graph includes a Z (Kolmogonov-Smirnov) statistic exploring differences between the two frequency distributions.

CHAPTER SEVEN

SYNTHESIS AND FUTURE DIRECTIONS

7.1. BRIEF SUMMARY

This thesis focused on quantifying mortality rates and identifying factors responsible for mortality in the early life of *Sepioteuthis australis*. The early life is defined as the embryo and hatchling ('paralarval') phases and as a result processes of mortality were investigated in these two stages independently. Chapters two through to five dealt exclusively with the embryonic phase whereas chapter six investigated the role of size-selectivity in the subsequent hatchling phase. Embryo mortality was defined as any individual that was unlikely to successfully hatch and therefore, included embryos that were unfertilised, had ceased, or were undergoing abnormal development. Given the species-specific differences in loliginid embryology (Baeg et al. 1992; Guerra et al. 2001); Chapter two) it was necessary to define the embryological process in *S. australis* in order to set a baseline for inter- and intra-specific comparisons, and to identify 'abnormal' embryos (Chapter two). Once the embryological scheme was established the direction of research adopted a hierarchical approach, investigating factors contributing to early mortality from;

(1.) A broad environmental perspective,
(2.) The micro-environment of the aggregated egg mass, and
(3.) Individual egg quality.

7.2. SYNTHESIS:

Throughout the course of this study embryo mortality in *Sepioteuthis australis* ranged from 2 to 25% and varied both spatially and temporally. Estimates of 25% exceed existing mortality rates for loliginid embryos developing in stable laboratory conditions (<10%, *S. lessoniana* Segawa 1987; *S. australis* Triantafillos 2001) and in the wild (c. 5%, *Loligo gahi* (Arkhipkin et al. 2000). From the collective results it can be broadly stated that embryo mortality and offspring survival is determined by when and where eggs are laid. This statement holds true on a number of cascading levels. For example, the mother and clutch the egg originates from; when and where the egg mass is attached; the relative position of the

embryo within the mass; and the sequence of hatching all contribute to offspring survival (Fig 7.1). Such inter-related processes can operate at different strengths potentially explaining the variation in mortality rates observed in this study.

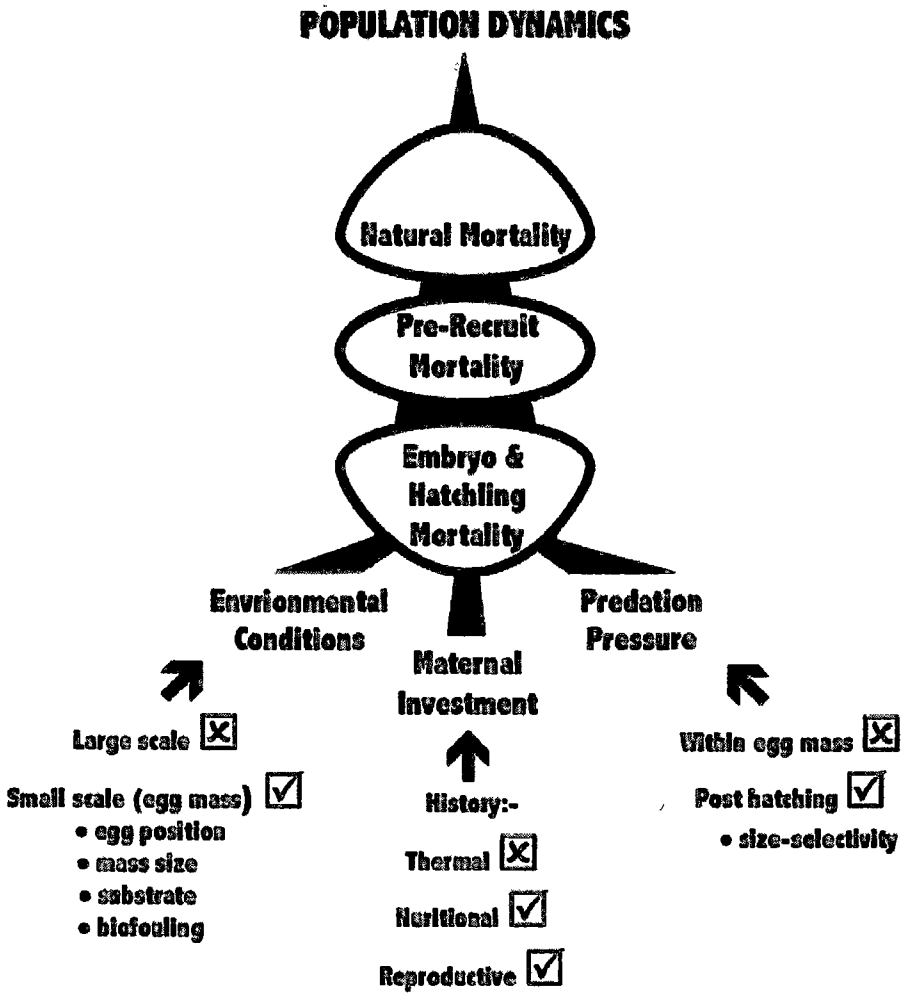


Figure 7.1. Further partitioning of natural mortality process in *Sepioteuthis australis* incorporating major findings. ☒ Signifies little to no contribution to early mortality, ☑ signifies positive contribution.

Maternal allocation of yolk resources is an important factor in offspring survival as it provides the necessary components to fuel development within the egg, whilst also allocating extra reserves to buffer the transition from embryo (endogenous) to hatchling (exogenous) (Vidal et al. 2002). Results from a component of this study (Chapter five) suggest that mature females with a poor nutritional history do not provide the embryo with sufficient resources that are

important for successful embryo development. Food stressed females produced smaller eggs of lower quality, a trade-off also exhibited in Baltic herring (Laine and Rajasilta 1999). The consequences of this response are two-fold; (1) it directly increases embryo mortality rates (Chapter five) and (2) it produces smaller hatchlings, compromising their competitive edge in comparison to larger conspecifics (Chapter six). Life history theory suggests that in a food limited environment females tend to offset early mortality by reducing fecundity and maintaining egg quality (Roff 1992). This however, may not be the case if resources are severely stressed, as hypothesised in this laboratory study. In addition, having a spawning mode where multiple clutches are laid over a relatively short spawning season can increase reproductive stress, leading to a sequential decline in offspring viability. From this component of research it can be suggested that large eggs spawned in an initial clutch, produced from a well nourished female, will inherently contain the necessary components for successful development. However, the chances of survival will subsequently depend on when and where the eggs are laid.

The 'egg packaging' strategy exhibited in the loliginids suggests high survival rates (Boletzky 1994). However, on closer inspection this strategy can be detrimental through constraining the developmental process and potentially suffocating the embryos (Chapters two to four). Depending on where eggs are laid, embryos will have varying chances of survival despite their high level of capsular protection (Boyle and Boletzky 1996). Embryos located deep within the interior of the egg mass are at a higher risk to mortality and this risk increases proportionately to egg mass size (Chapters three and four). Communal spawning is a strategy exhibited by the majority of loliginids (Griswold and Prezioso, 1981; Sauer et al. 1992; Mohamed 1993; Wada et al. 1995) and for some species egg masses can exceed 12m (40 feet) in diameter (McGowan 1954). It is likely that such large masses would exhibit unfavourable areas within the mass potentially promoting higher mortality rates than those observed in *S. australis*.

Natural temperature fluctuations recorded during the course of this study had no clear effect on embryo mortality rates, but governed rates of development (Chapter two and three). This is typical for most invertebrates, where developmental rates are inversely proportional to incubation temperature (McMahon and Summers

1971; Clarke 1982; Boletzky 1994; Caveriviere et al. 1999). Similarly the position of the embryo within the egg strand affected rates of development, with embryos located at the proximal ends exhibiting retarded developmental rates compared to distal embryos. The magnitude of these within-strand differences was also governed by temperature (Chapter three), along with the strand's relative position within the mass (Chapter two). Although temperature had no direct effect on embryo mortality, it, along with the position of the embryo within the mass, indirectly reduces the hatchlings' chances of survival by influencing hatchling size. Therefore, incubation temperatures, the constraining properties of the egg mass, and the previously mentioned role of maternal effects, contributes to considerable variation in size at hatching. Variation in hatchling size has been documented in other loliginids such as *Sepioteuthis lessoniana* (Ikeda et al. 1999) and *Loligo vulgaris* (Villanueva et al. 2003), and as such may also have significant flow on effects in terms of size-selective mortality processes (Chapter six).

7.2. Potential Applications and Future Research Directions

Obviously numerous other inter-connecting biotic and abiotic processes, not covered in this thesis may contribute to early mortality rates in *Sepioteuthis australis*. The results obtained, however, offer preliminary insights into a field that has not been directly addressed. The identification of factors that significantly contribute to natural embryo mortality rates is an important first step in understanding *S. australis* and other loliginid life histories. With further refining and quantification on broader temporal and spatial scales, early mortality 'indices' can be established and incorporated into stock-recruitment models assisting fisheries management.

To obtain mortality indices that can potentially be used in stock-recruitment models, three main areas were identified that need to be independently addressed and further refined;

(1.) The flow on effects of maternal condition over an extended spawning season,

(2.) Further investigation of mortality processes within the egg mass examining the role of biotic and abiotic factors on larger temporal and spatial scales,

(3.) Investigation of size-selective mortality processes though application of the technique described in chapter six to all post-hatching life stages

7.2.1. Maternal Effects

Recent research on *Sepioteuthis australis* has indicated that significant differences in ovulated egg sizes exist over the spring/summer spawning season (Jackson and Pecl 2003). Such extreme differences in egg size over a relatively short period remains unexplained but suggested to be in response to environmental or physiological influences. Jackson and Pecl (2003) further suggests that *S. australis* exhibits little evidence of feeding on the spawning grounds, a finding concurrent with *Loligo vulgaris reynaudii* (Augustyn 1990). It is therefore possible that observed changes in egg size are potentially reflecting the females' nutritional and reproductive history (as seen in Chapter five). It would, therefore, be beneficial to explore whether correlates exist between egg size and female age, condition and local productivity. The flow on effect of such changes in terms of egg quality and viability would be of particular interest and it may be theoretically possible to use female attributes coupled with environmental data to predict hatching success.

7.2.2. Large scale spatial and temporal quantification of embryo mortality

For loliginids that spawn benthic eggs in shallow water, conducive to *in situ* investigations, quantifying embryo mortality rates are feasible. Accompanying egg density surveys, which currently aim to provide a 'quick and easy' assessment of the spawning stock (Sauer et al. 1993; Moltschaniwskyj et al 2002), with a structured sampling protocol investigating mortality rates will provide valuable information aiding stock-recruitment models. Expanding such protocols over larger spatial and temporal scales and simultaneously measuring other environmental factors such as salinity, dissolved oxygen, sedimentation and UV radiation, will provide further information on the direct and indirect processes responsible for embryo mortality.

7.2.3. Examine size-selectivity over multiple life stages

The statolith technique used in chapter six to investigate the 'bigger is better' hypothesis yielded promising results, indicating a potential for widespread application in cephalopod fisheries management. Further research is required, however, to refine this method and reduce some of the variability incorporated within the model. Efforts should be made to investigate whether the natal area of the statolith is a better predictor of hatchling size than simple linear dimensions, as suggested by Meekan et al. (1998). Furthermore, the model needs to be re-tested, eliminating the potential for year-to-year variation that may have been incorporated in this study. The major benefit of this method, however, is the fact that it can be applied to any life stage of a squid, providing the hatchling statolith and somatic relationship is validated and the natal ring is preserved in the species. Through collecting hatchlings, juveniles, maturing adults and successfully recruited adults from the same cohort and applying this statolith technique it is theoretically possible to identify what life stage is most susceptible to size-selective mortality processes.

There are still many unanswered questions relating to early mortality rates. Studies such as those outlined above may shed further light on the patterns and processes of mortality during the early 'vulnerable' stages. In quantifying these processes over large spatial and temporal scales it will be possible to reduce some of the variability encompassed within existing stock-recruitment relationships.

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