Reproduction of the South African abalone, *Haliotis midae*

by
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Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the authorship owner thereof (unless to the extent explicitly otherwise stated) and that I have not previously, in its entirety or in part, submitted it for obtaining any qualification.

Date: 23 November 2011

Adelle Roux

Abstract

Currently, South African aquaculture is dominated by the cultivation of *Haliotis midae*, which is estimated as the most lucrative sector of the industry, with 934 t being export in 2008, totalling an income of ZAR 268 million (40 million USD) in 2008. This represents 81% of the total rand value of the aquaculture sector. Abalone was the highest aquaculture commodity exported during the last two years from South Africa, representing 24% of the total tonnage exported. Employment in the aquaculture sector increased by approximately 80% between 2005 and 2008, and was highest in the abalone sector where the number of people employed increased by 234%.

Despite these high production rates, no hatchery procedures have been developed specifically for *H. midae*. Most procedures and protocols currently used in South African abalone hatcheries have been adopted from cultivation methods used for foreign species. Although certain aspects of reproduction are universally conserved between abalones, it is important to consider the physiology and the origin of the species studied.

To date, no scientific research has been conducted on the reproduction of *H. midae*, except for a few studies in the early 1990s, which focused on the basic reproduction of this species. No further studies have been done on *H. midae* reproduction under intensive culture. Currently, hatch-out rates obtained by most abalone farms in South Africa averages 80%, with a 50% settlement rate, and a final hatchery output of only 30%.

This study reports on various aspects of *H. midae* reproduction that can influence its commercial culture. A detailed histological characterisation of gametogenesis was developed. Findings indicated that cultured *H. midae* reaches 50% sexual maturity at a shell width of between 25 mm and 30 mm.

During fertilisation trials, a sperm concentration of 50 000 sperm mL^{-1} and egg concentrations lower than 50 eggs mL^{-1} produced the highest hatch-out rates. Whilst fertilisation volume did not influence fertilisation success, fertilisation potential of the eggs did decrease with time. Eggs older than 100 minutes showed a lower fertilisation potential than eggs fertilised earlier.

A larval stress test was developed to evaluate larval resistance against chemical stress. It was determined that 50% of resultant larvae would exhibit morphological abnormalities after fertilised eggs were incubated in 0.7% dimethyl sulfoxide (Me_2SO) for a 24 hour period. If larvae exhibited fewer abnormalities at this concentration, it could be deduced that the larvae had a high resistance to the negative effect of the toxicant, and could thus be seen as good quality larvae.

When evaluating hybridisation potential between *H. midae* and *H. spadicea*, it was found that it was possible to fertilise spawned *H. midae* eggs with biopsied *H. spadicea* sperm.

By incorporating the results obtained from the present study into current hatchery systems on South African abalone farms, higher hatchery yield could be achieved, which in turn would lead to an increase in commercial revenue.

Opsomming

Die Suid-Afrikaanse akwakultuur sektor word tans oorheers deur die produksie van *Haliotis midae*, en word gereken as die mees winsgewendste bedryf in die industrie, met 934 t uitgevoer in 2008, na-raming ‘n inkomste van ZAR 268 miljoen (40 miljoen Amerikaanse doller). Dit verteenwoordig sowat 81% van die totale rand waarde in die akwakultuur bedryf. Perlemoen was ook die grootste uitvoer kommoditeit gedurende die laaste twee jaar, en het tot sowat 24% van die totale uitvoer vanuit akwakultuur bygedra. Werksgeleenthede in die akwakultuur sektor het tussen 2005 en 2008 met ongeveer 80% gegroei, waarvan die hoogste groeisyfer in die perlemoen bedryf was, waar die aantal werknemers met 234% toegeneem het.

Ten spyte van hierdie hoë produksie omset, is daar tans geen protokolle wat spesifiek vir die produksie van *H. midae* ontwerp is nie. Meeste van die tegnieke wat huidiglik gebruik word op Suid-Afrikaanse plase, is gebaseer op, en aangepas vanaf metodes daargestel in die internasionale bedryf vir uitheemse spesies. Alhoewel sekere aspekte van reproduksie tussen perlemoen spesies verband hou, is dit belangrik om die fisiologie en oorsprong van die spesie van belang, in ag te neem. Wetenskaplike navorsing gedoen op die reproduksie van *H. midae* is beperk tot studies in die vroeë 1990s, wat die basiese beginsels van die spesie se reproduksie ondersoek het. Daar is geen verdere studies op die reproduksie van *H. midae,* veral onder intensiewe teel toestande, gedoen nie. Tans toon die meeste perlemoen plase in Suid-Afrika ‘n produksie persentasie van ongeveer 80% larwes vanaf bevrugting, met ‘n afname na 50% met vestiging en ‘n gevolglike uitset van slegs 30%.

Hierdie studie doen verslag oor verskeie reproduksie aspekte van *H. midae* wat die teel doeltreffendheid van perlemoen op kommersiële plase kan beïnvloed. ‘n Gedetaileerde histologiese karakterisering van gametogenese is ontwikkel. Daar is gevind dat geteelde perlemoen 50% geslagsrypheid bereik met ‘n skulp wydte van tussen 25 mm en 30 mm.

Tydens bevrugting eksperimente is bepaal dat 50 000 sperm mL^{-1} en ‘n eier konsentrasie van laer as 50 eiers mL^{-1}, die optimale gameet konsentrasies is vir effektiewe bevrugting. Alhoewel die volume water waarin bevrugting plaasvind nie ‘n invloed getoon het op bevrugtingsukses nie, is daar wel gevind dat die eiers se potensiaal om bevrug te word, afneem met verloop van tyd. Eiers ouer as 100 minute het ‘n verlaagde bevrugtingspotentiaal getoon teenoor eiers wat vroeër bevrug is.

‘n Larwale stres toets is ontwikkel om larwale weerstand teen chemiese stres te bepaal. Daar is gevind dat 50% van geproduseerde larwes morfologiese abnormaliteite sal toon indien bevrugte eiers vir ‘n periode van 24 uur in 0.7% dimetiel sulfoksied (Me_2SO) geïnkubeer word. Indien larwes minder abnormaliteite toon by hierdie konsentrasie, beteken dit dat hierdie larwes meer weerstand kan bied teen die negatiewe effek van die toksiese middel, en dus beskou kan word as goeie kwaliteit larwes met hoë lewensvatbaarheid.

Met die evaluering van hibridisasie potensiaal tussen *H. midae* en *H. spadicea*, is gevind dat dit moontlik is om vrygestelde *H. midae* eiers te bevrug met *H. spadicea* sperm wat verkry is deur ‘n biopsie.

Die implementering van hierdie studie se bevindinge in kommersiële *H. midae* produksiesisteme sal daadwerklik bydra tot die optimisering van bestuurspraktyke en ‘n verhoging in die totale produksie doeltreffendheid van sulke sisteme.

Acknowledgements

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Chapter 1 Introduction

1.1. Reproductive biology of abalone

Abalones form part of the phylum Mollusca, which comprises approximately 50 000 extant and 35 000 extinct species. It is a very diverse group, which includes species such as chitons, snails, abalones, oysters, and octopuses (Newell 1970). Haliotids belong to the order Archaeogastropoda, which is the oldest and most primitive group of prosobranch gastropods (Killburn & Rippey 1982; Muller 1986).

1.1.1. General anatomy

Abalones are characterised by a round or ear-shaped shell with respiratory pores along the left margin of the shell that successively close during growth (Muller 1986; Genade *et al.* 1988; Hahn 1988). Beneath the shell lie the head, a large muscular foot, and the visceral mass, all of which are attached to the shell by the adductor muscle (**Figure 1.1**). The foot, being the edible part of the abalone, is girdled by the mantle and epipodia, and can account for more than one third of the animal's weight (Fallu 1991).

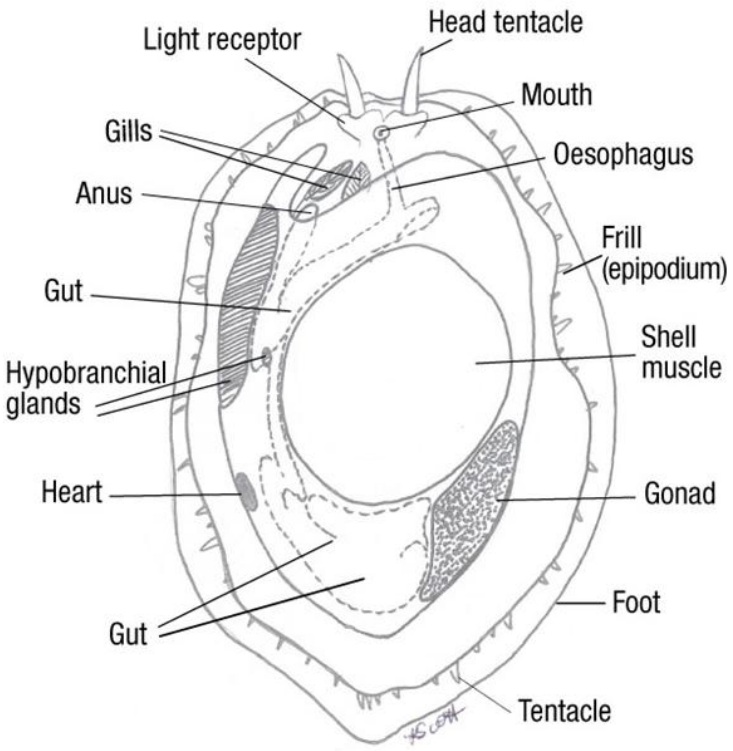

Figure 1.1 Ventral view of the anatomy of the abalone (Scott 2008).

1.1.2. Reproductive anatomy

The gender of an abalone can be determined by turning it over and gently pushing the fringe-like epipodia at the right rear-side of the body away from the shell. This will expose the coloured gonad, which can be seen through the connective tissue of the mantle. The digestive gland is enveloped by the single gonad to form the conical appendage (**Figure 1.2**). This structure constitutes the bulk of the visceral mass and develops along the right side of the body and around the right posterior margin of the adductor muscle (Newman 1966, 1968, 1969; Hahn 1988; Hooker & Creese 1995).

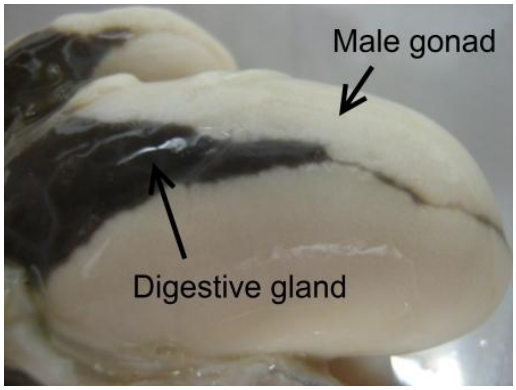

Figure 1.2 The conical appendage of *Haliotis midae* indicating the gonad-enveloped digestive gland.

Female *Haliotis midae* can be identified by a dark green ovary while males are distinguished by a cream coloured testis (**Figure 1.3**). In other abalone species, the female gonad can vary in colour, ranging from brown (*H. iris*) (Wilson & Schiel 1995), to green or blue-green (*H. asinina* and *H. australis*) (Shepherd & Laws 1974; Capinpin *et al.* 1998).

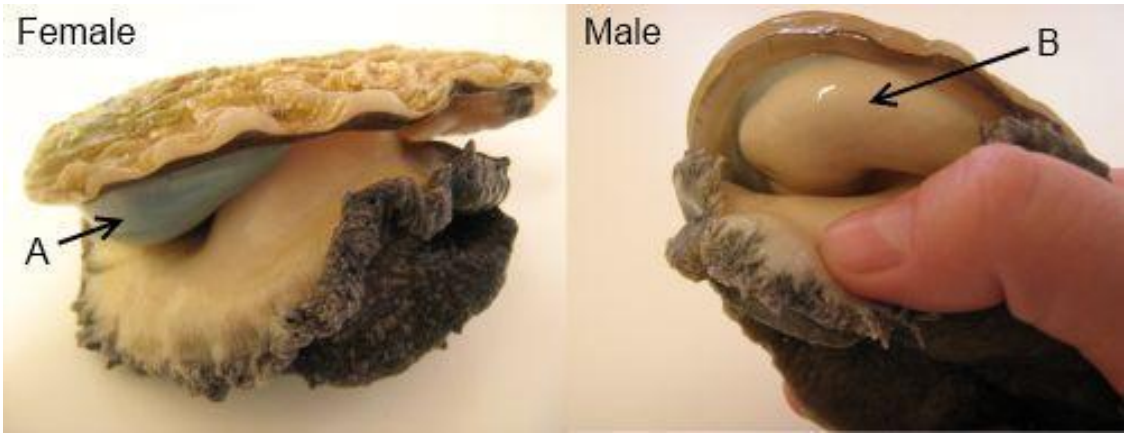

Figure 1.3 Gender identification of *Haliotis midae*. A = green coloured female gonad; B = cream coloured male gonad.

The ovary consists of a series of compartments, separated by trabeculae (Newman 1967; Hahn 1988). These trabeculae comprise of sheets of connective tissue that support the germinal epithelium; the site of oogenesis (**Figure 1.4**). The lumen of a fully developed ovary is filled with large eggs, embedded in a gelatinous matrix, before spawning (Newman 1967).

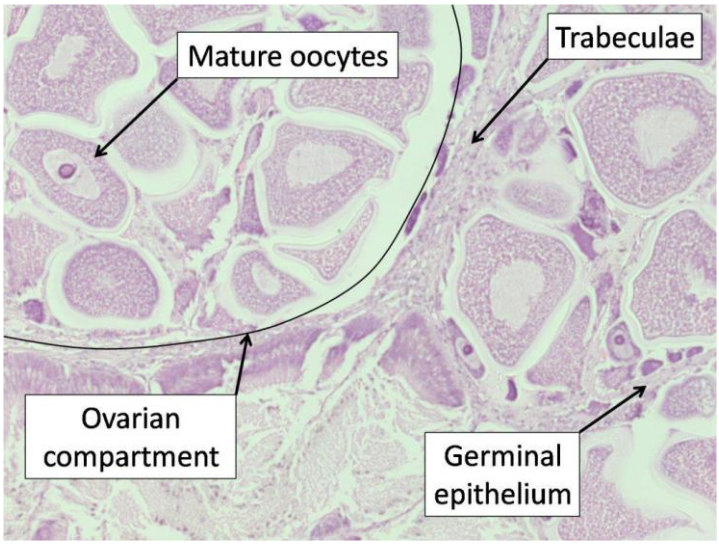

Figure 1.4 Histological cross-section of the female gonad of *Haliotis midae*.

In the male, the lumen of the testis is traversed by tubules of connective tissue lined with germinal epithelium where spermatogenesis takes place (**Figure 1.5**). When fully mature, the testis lumen is filled with spermatozoa ready for spawning.

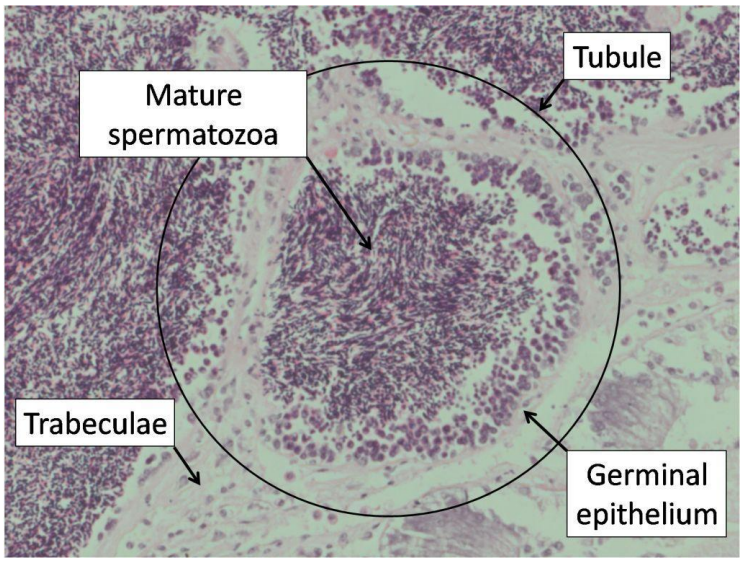

Figure 1.5 Histological cross-section of the male gonad of *Haliotis midae*.

1.1.3. Reproductive cycle

1.1.3.1. Spawning

Abalones are dioecious broadcast spawners and fertilisation occurs externally (Tarr 1989). Having a primitive reproductive system, the reproductive strategy of abalones is limited to the emission of large numbers of gametes into the environment, with no protection or special provision to the developing larvae (Purchon 1977). Mature abalones enter regular reproductive cycles. These cycles vary between species and are largely influenced by water temperature, which affects gonad maturation (Litaay & De Silva 2003; Awaji & Hamano 2004; Grubert & Ritar 2004). The cycles are synchronised in a population in order to benefit from simultaneous mass spawnings, as successful fertilisation in broadcast spawners is largely dependent on the number of released gametes (Hahn 1988).

Spawning begins when the shell is lifted to a degree where the gonad is visible and the adductor muscle contracts, rapidly compressing the conical appendage between the foot and the shell (**Figure 1.6**). This results in the gametes being released via a single gonad duct directly into the kidney. From there the gametes are forced out the respiratory pores into the surrounding seawater via the nephridiopore duct (Hahn 1988). Males usually spawn first, stimulating the females to release their eggs. The eggs sink through the sperm cloud because of their negative buoyancy (Huchette *et al.* 2004). For this reason, it is common for female abalones to position themselves on high areas on the reef (Newman 1967; Hahn 1988; Fallu 1991). During the process of spawning, mature eggs are extruded loose from each other, while immature eggs are released as coagulated masses (Hahn 1988). Eggs slowly expand after spawning, probably due to hydration of the egg content (Huchette *et al.* 2004).

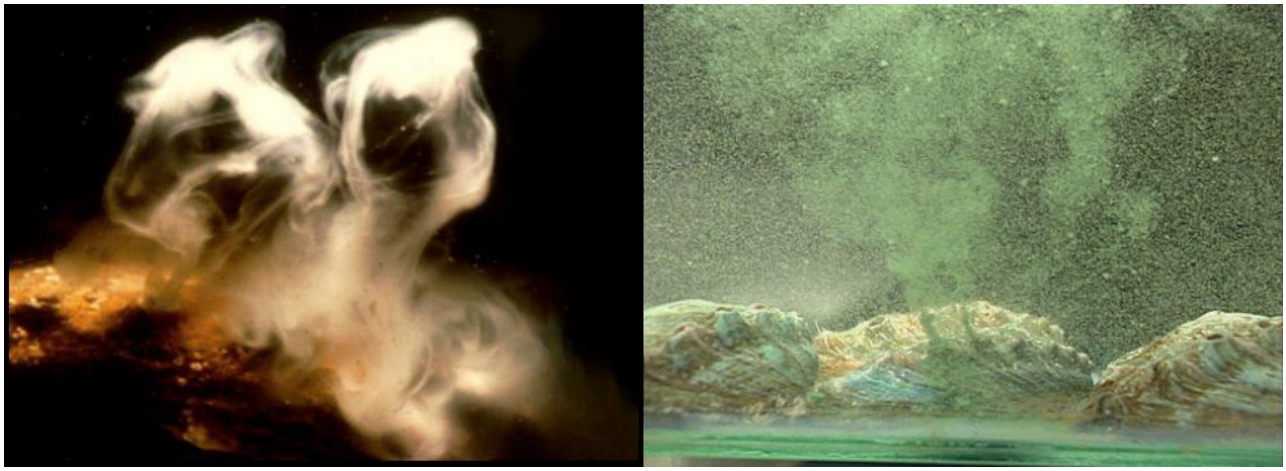

Figure 1.6 Spawning observed in a *Haliotis rufescens* male (left) and females (right) (Friesen 1977).

1.1.3.2. Fertilisation

During fertilisation, abalone sperm reach the egg vitelline layer after penetrating the gelatinous layer. The vitelline layer comprises of carbohydrates, proteins, and glycoprotein fibres, which are held together by hydrogen bonds. Upon reaching the vitelline layer, an exocytotic acrosome reaction is induced during which the sperm releases a 16-kDa non-enzymatic cationic protein called lysin onto the surface of the vitelline layer. Lysin dimers bind species-specifically to a vitelline envelope receptor for lysin (VERL) on the vitelline membrane. The NH_2-terminal of lysin is mostly involved in the specificity of this binding, while the COOH-terminal may be involved in the dissolution of the vitelline membrane (Lewis *et al.* 1980, 1982, 1989; Huchette *et al.* 2004). After binding, lysin causes the vitelline envelope fibres to unravel and splay apart (**Figure 1.7**), creating an opening in the vitelline layer where sperm can pass through into the egg cytoplasm (Kresge *et al.* 2001; Galindo *et al.* 2002).

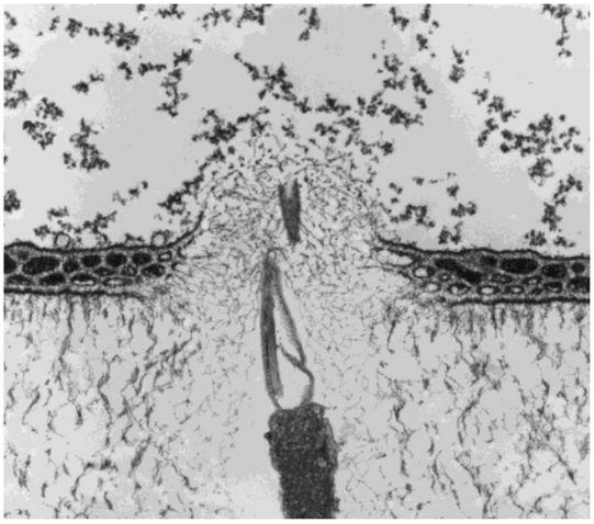

Figure 1.7 Transmission electron micrograph (X 26 000) depicting an abalone sperm penetrating the egg vitelline layer (Kresge *et al.* 2001).

Fusion of the male and female gametes activates the contractile cytoskeleton of the egg to draw the sperm into the cytoplasm after which the male and female pronuclei fuse and the egg begins cleavage (Kresge *et al.* 2001). Successful fertilisation in abalone is dependent on various factors, such as gamete concentrations, egg viability and fertilisation volumes. Studies in several abalone species have shown that fertilisation in abalone is not effective at all sperm concentrations (Clavier 1989). Low hatch-out success at low sperm concentrations is due to a decreased probability of successful egg-sperm encounters, while high sperm concentrations could result in polyspermia (Grubert 2005). Fertilisation also needs to occur during a certain period after spawning, as egg viability has been shown to decrease over time (Baker & Tyler 2001; Zhang 2008).

1.1.3.3. Larval development

The process of larval development of most abalone species is similar and takes 4 to 10 days, depending on the species and water temperature (Hahn 1988). Temperature is known to play an important role in fertilisation success, the onset of hatch-out and other early life stages of abalone. Larval development occurs at a faster rate in warmer water, but is terminated at high temperatures, while sub-optimal temperatures result in a longer larval phase (Hahn 1988). Larval stages have been defined for *H. laevigata, H. rubra*, *H. tuberculata*, and *H. midae* (**Table 1.1**). Following hatch-out, the ciliated trochophore larvae moves actively in the water column. Shortly after this, shell formation occurs, resulting in veliger larvae.

Table 1.1 Larval stages and development (in hours) at set temperatures for *Haliotis rubra* (16.9°C), *H. laevigata* (16.4°C), *H. tuberculata* (23°C) and *H. midae* (18°C) (Grubert 2005; Courtoise de Vicose *et al.* 2007; present study).

Stage	***H. tuberculata***	***H. midae***	***H. rubra***	***H. laevigata***
Fertilisation	0h00	0h00		
First polar body	0h25	0h40		
Second polar body	0h40	1h00		
First cleavage (2 cell)	0h50	1h30		
Second cleavage (4 cell)	1h25	2h00		
Third cleavage (8 cell)	1h35	3h30		
Fourth cleavage (12 cell)	1h55			
Fifth cleavage (16 cell)	2h00	4h00		
Morula	2h30	5h00		
Blastula	3h15	6h00		
Gastrula	4h30	7h00		
Appearance of cilia	6h20	8h00		
Stomodeum	7h20	10h30		
Completion of protochal cilia	8h20	11h00	10h00	4h00
Hatch-out of trochophore larva	9h10	12h00	18h00	20h00
Larval shell formation	9h35	16h00		
Veliger larva with complete velum	11h45	18h00	22h00	28h00
Formation of integument attachment	15h20	20h00	38h00	42h00
Development of foot mass	15h20	24h00		

1.1.3.4. Settlement

The veliger stage is followed by settlement, metamorphosis, and the deposition of the peristomal shell. Settlement occurs a week to a month after the veliger stage, depending on the species and ambient conditions (Hahn 1988). The shift from pelagic larvae to benthic adult form is induced by interactions between settling larvae and their benthic environment. Factors affecting larval development prior to and during metamorphosis can significantly affect adult survival rates (Williams & Degnan 2009). After settlement, young abalones (spat) begin to graze on diatoms that cover rocks and encrusting coralline alga (e.g. *Lithothamnion* spp.). When *H. midae* juveniles are large enough, they begin to feed by trapping kelp fronds. Trap-feeding is an energetically economical method of feeding, and is accomplished by raising the front section of the body and extending the foot forward. When a piece of seaweed washes up against the foot, the animal will clamp down and trap the kelp (Tarr 1989).

1.2. Abalone species of South Africa

Five species of the genus *Haliotis* are found along the southern coastline of South Africa, with a sixth species, *H. pustulata*, (**Figure 1.8**), occurring along the southern coast of Mozambique (Muller 1986).

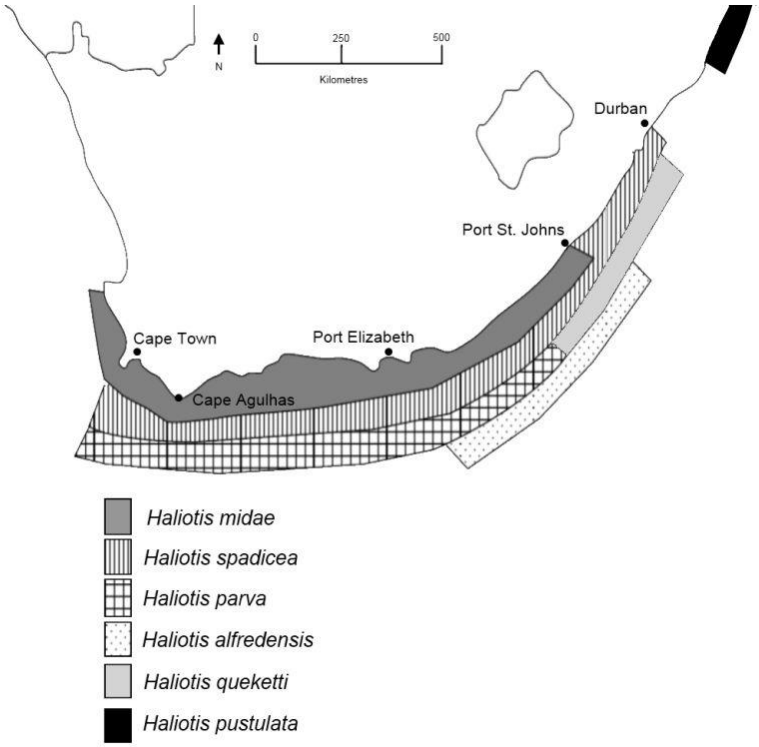

Figure 1.8 Distribution of *Haliotis* species along the coastline of South Africa (Lindberg 1992).

These abalone species are classified according to differences in shell shape and colour, as well as number of whorls and ridges (**Figure 1.9**). The smallest of these species, *H. alfredensis, H. pustulata* and *H. queketti* (ranging from 30 mm to 70 mm in size) are quite rare, and occur along the eastern coast of Southern Africa at depths to 160 m. The three remaining species, *H. parva*, *H. spadicea* and *H. midae*, are more commonly found along the south coast of South Africa. Of these three species, *H. parva* is the smallest, with an average size of 50 mm (Muller 1986).

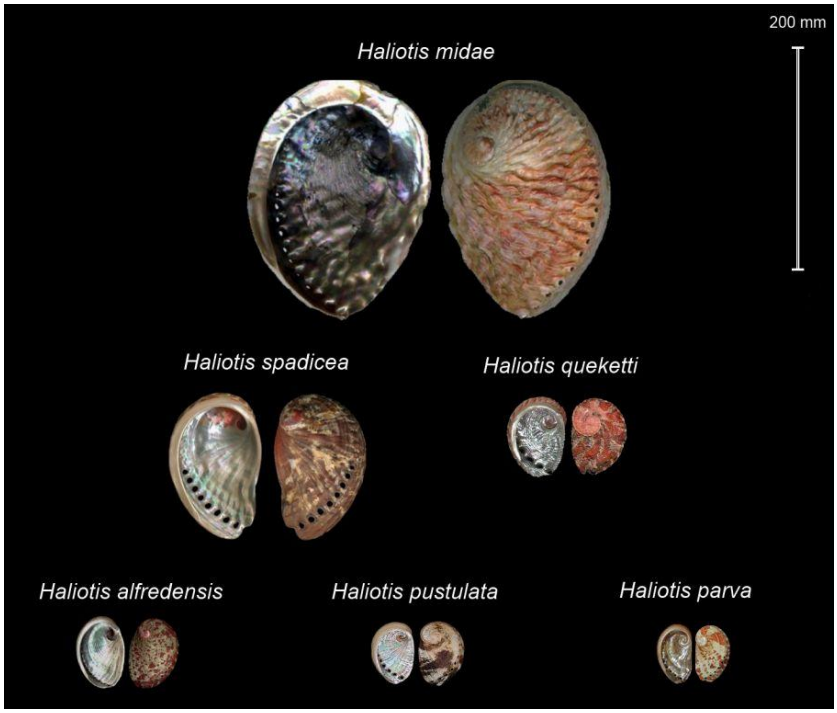

Figure 1.9 Shell morphology and relative sizes of *Haliotis* species found along the coastline of South Africa (Source: www.gastropods.com).

1.3. History of abalone fisheries in South Africa

Haliotis midae, also known as perlemoen, is currently the only cultivated species in South Africa (Troell *et al.* 2006; Raemaekers & Britz 2009). The name “perlemoen” is derived from the Dutch word for mother-of-pearl, literally translated as *paarl* being pearl and *moer* translating as mother (Tarr 1989).

Abalone shell middens have been found in areas extending from the western coast to the southern coast of South Africa. It is estimated that most of these middens date back to between 2 000 and 3 500 years, while world-wide, the earliest middens date back more than 125 000 years AD. The occurrence of South African middens is attributed to the food-searching activities of “Strandlopers”, an

indigenous tribe of South Africa, which cleaned the animals on the beaches to avoid carrying unnecessary weight back to their campsites or caves (Tarr 1989).

Today, the greatest demand for abalone is from the East (Tarr 1989; Britz *et al.* 2009). This demand was the reason for the establishment of an abalone industry in South Africa. In 1949, wild harvesting of abalone for the export market started in the Gansbaai area and expanded to the west and east coast. In 1953, harvesting was estimated at 770 t (meat weight); all of which were canned and exported to the Far East (Tarr 1989). Due to overfishing in this period, a quota system was implemented in 1968 and revised in 1983, allowing a whole weight quota of 660 t (Tarr 1989). Harvesting was also regulated by a minimum legal size (MLS) of 138 mm shell length and 114 mm shell width (Tarr 1989; Troell 2006). Despite this regulation and the application of a strict quota system, stocks continued to decline due to severe poaching. In 2004, commercial harvesting quotas were reduced from 660 t to 230 t (Tarr 1992, 1995, 2000; Day & Branch 2002). In 2007, government added *Haliotis midae* to Appendix III of the Convention on International Trade in Endangered Species (CITES), thus preventing the export of abalone without a permit.

As none of the implemented measures rebuilt the natural stock, government took severe steps and banned all commercial abalone fishing in 2008. This affected more than 1 000 local anglers that depended on abalone harvesting as a livelihood. On 1 July 2010, the two-year-old ban on commercial abalone fishing was lifted and plans set in motion to assist local anglers as well as protect abalone resources (Source: www.fis.co.za).

1.4. History of abalone cultivation in South Africa

Government restrictions on harvesting natural stock and increased poaching have been the leading motivations for abalone cultivation in South Africa (Fleming & Hone 1996; Troell *et al.* 2006). Farming was initiated in the early 1990s after the successful spawning and production of viable spat and juveniles of wild-caught *Haliotis midae* (Genade *et al.* 1988). Initial research indicated that growth rates in captivity were enhanced compared to that in the wild, and cultivation activities rapidly expanded. In 2003, it was estimated that South Africa cultivated over 500 t of abalone, and by 2008, this number increased to approximately 850 t. The most recent survey reported an export of 934 t, making South Africa the second largest supplier of cultivated abalone in the world aside from China (Troell *et al.* 2006; Britz *et al.* 2009).

Commercial cultivation of *H. midae* has been described as the pioneer industry of mariculture in South Africa, and is considered the most lucrative in the aquaculture sector. By 2009, 22 licensed commercial farms had been established, although not all of these are registered for the export trade. Five more farms are scheduled for development (Britz *et al.* 2009). Most of the established farms are

located along the coastline of the Western Cape Province, but others have been established as far north as Port Nolloth, and east as Port Elizabeth (**Figure 1.10**).

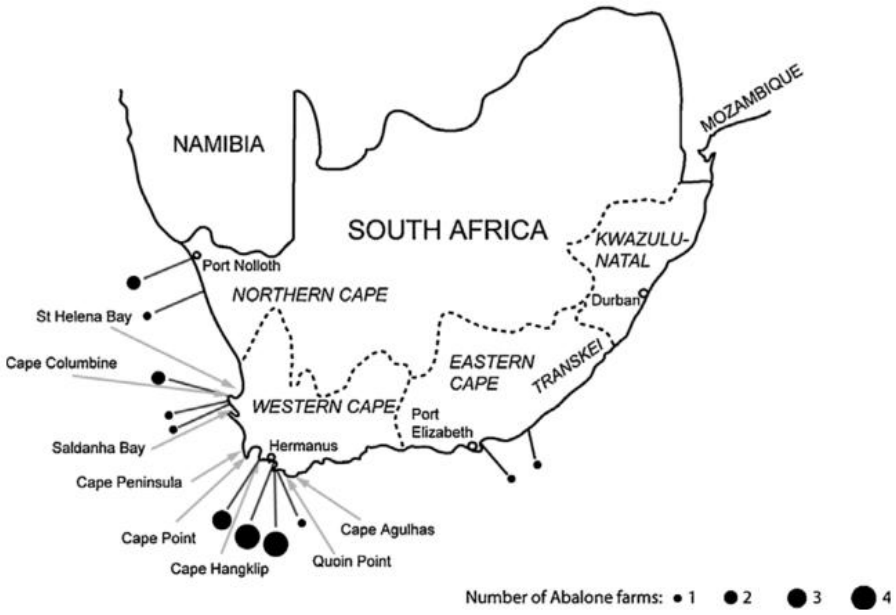

Figure 1.10 Established abalone farms around the coast of South Africa (Troell *et al.* 2006).

1.5. History of reproductive work on *Haliotis midae*

Research on the reproduction of *Haliotis midae* started in 1966 by Newman, who investigated the movement, reproduction, and growth of wild animals. His studies focused on the gonad structure and histology, sex ratio, sexual maturity, spawning cycle and fecundity of *H. midae* occurring at three localities; Stony Point (west of Hermanus), Sea Point (west of Cape Town) and Dassen Island (south of Saldanha Bay). Results indicated that males and females were present in a 1:1 ratio and that 100% sexual maturity was reached at a shell width (SW) of 100 mm with spawning occurring twice a year (April and September) in certain areas (Newman 1966, 1967, 1968, 1969). A linear relationship was also found between animal weight and fecundity with a female of 115 mm SW producing 4.5 million eggs per spawning, while a female of 160 mm produced more than 15 million eggs per spawning.

Wood and Buxton (1996) later described histological structures of *H. midae* gonads and concluded that the process of gametogenesis was similar to that of other *Haliotis* species. The study also contradicted earlier results, showing that the onset of sexual maturity occurs at 20 mm SW with 50% maturity attained at 35 mm. This indicated that animals reached 100% sexual maturity at 50 mm, instead of 100 mm as reported by Newman (Newman 1967; Wood & Buxton 1996).

In 1981, the first attempt was made to culture wild-caught *H. midae* after collection of animals in the area between Hangklip and Quoin Point, South Africa, during August and September. The animals were transported to a laboratory where ten animals were placed per 1 600 L tank. The following morning spontaneous spawnings occurred due to the stress of handling (Genade *et al.* 1988). Although the number of animals contributing to the spawning could not be determined, fertilisations were done by siphoning the eggs and adding sperm at a concentration of 120 000 mL^{-1}. Settlement was successfully induced by using γ-aminobutyric acid (GABA) following the experimental design of Morse (1989). In 1985, another experiment to induce spawning was conducted after collection of animals during May and June (Genade *et al.* 1988). The same husbandry was followed as during the abovementioned trial and spawning was successfully induced using ultraviolet (UV) treated water and elevated temperatures (Chen 1984). Fertilisation procedures were similar in both trials, but the second resulted in poor fertilisation rates (i.e. 11% to 25%) in comparison to the first trial (80%). The reason for this was concluded to be the lack of ripeness of the gonads, and it was suggested that methods should be developed to condition *H. midae* to ensure the availability of mature gametes throughout the year.

The studies by Newman (1966, 1967, 1968, 1969), Genade *et al.* (1988), and Wood and Buxton (1996) set the groundwork for the culture of *H. midae*. To date however, no further research has been conducted to verify or elaborate on these findings, with current hatchery systems on South African farms adapted from overseas industries.

1.6. Procedures followed on South African abalone farms

1.6.1. Husbandry

Current broodstock on South African abalone farms were wild-harvested in the mid 1990s, with most animals obtained having similar shell lengths. Although the age of these animals is not known, when considering shell lengths, it can be estimated that most of the broodstock are approximately 15 years old, with an average length and weight of 20±5 cm and 1.5±0.5 kg, respectively.

Male and female broodstock are kept in separate tanks at a density of five animals per tank. The 40 L plastic tanks are rectangular and are supplied with air and filtered (1 µm) sea water (FSW) at a flow rate of 90 L h^{-1}. Animals are kept in isolated rooms at a holding temperature of 18°C with photoperiods of 12 hours light and 12 hours dark. The tanks are cleaned every third day after which the animals are supplied with *Ecklonia maxima* or *Laminaria pallida,* depending on availability (**Table 1.2**).

Table 1.2 Comparison of husbandry protocols for various cultivated *Haliotis* species (Fleming 2000).

Species	Tank volume (L)	Flow-rate (L min^{-1})	Holding temperature (°C)	Diet	Photoperiod (light:dark)
H. discus hannai	1 000	6.2	17 – 20	*Undaria pinnatifida*	
H. iris	1 000	11	Ambient	*Macrocystis pyrifera*	12L : 12D
H. laevigata	3 000	11.7	16	*Rhodophyta, Phaeophyta, Chlorophyta*	12L : 12D
H. midae	40	1.5	18 – 19	*Ecklonia maxima*	12L : 12D
H. rubra	800	8	18	*Ulva lactuca*	Natural
H. rufescens	227		Ambient	*Nereocystis luetkeana*	

1.6.2. Conditioning

New *H. midae* broodstock are selected for breeding based on physical appearances such as shell lengths and -widths as well as general health. These animals are selected at the age of three to four years (70 to 120 mm SL), when the gonads become externally visible. Although the young animals do spawn naturally at this age, they are not conditioned to spawn when chemically induced. To achieve this, animals are kept in isolation in broodstock rooms at a density of three animals per tank. During this period, which takes up to six months, the animals are treated the same as the established broodstock, being fed a diet of kelp and kept at 18°C in a 12 hour dark, 12 hour light system (**Table 1.3**).

Table 1.3 Comparison of broodstock conditioning methods for various *Haliotis* species (Fleming 2000).

Species	Average shell length (mm)	Stocking density (animals 1 000 L^{-1})	Period (weeks)	Temp (°C)	Feeding protocol
H. discus hannai	80	50	15	20	Daily
H. iris	> 100	90	14		Twice per week
H. laevigata	140	40	20 – 22	17	Every 3 days
H. midae	100	125	20 – 25	18	Twice per week
H. rubra	> 145	15	12 – 13	Ambient	Twice per week
H. rufescens	65 – 100		11 – 13	Ambient	As necessary

1.6.3. Spawning and fertilisation

Natural spawnings can be triggered by various environmental factors and are mostly dependent on season (**Table 1.4**). A sudden change in water temperature, exposure to air during low tide, photoperiod, lunar cycle, release of gametes from other individuals, surface winds, physical disturbances, food supply, genetic and hormonal factors, and heavy surf, can individually or in combination trigger spawning (Uki & Kikuchi 1984; Hahn 1989; Fallu 1991).

Table 1.4 Spawning season and frequencies of various *Haliotis* species (Hahn 1988).

	Summer (SH) Winter (NH)			Autumn (SH) Spring (NH)			Winter (SH) Summer (NH)			Spring (SH) Autumn (NH)		
Species	**Jan**	**Feb**	**Mar**	**Apr**	**May**	**Jun**	**Jul**	**Aug**	**Sep**	**Oct**	**Nov**	**Dec**
H. discus hannai									x	x	x	X
H. fulgens				x	x	x	x	x				
H. iris			x	x								
H. laevigata										x	x	x
H. midae				x					x	x		
H. roei			x	x	x	x	x	x	x	x	x	x
H. rubra			x	x	x	x	x	x	x			
H. rufescens	x	x	x	x	x	x	x	x	x	x	x	x

SH = Southern Hemisphere; NH = Northern Hemisphere.

On abalone farms, spawning can be induced by simulating natural spawning cues, or by means of chemical stimulation (**Table 1.5**). Increasing the water temperature of mature broodstock rapidly to 5°C above ambient temperature during the first 30 minutes, and then reducing the temperature again over a two and a half hour period, has been shown to induce spawning in certain species. This method is however not recommended as it also induces the release of immature gametes (Hahn 1988; Fallu 1991). By subjecting broodstock to a rapid change in water pH can also induce spawning, although this method is not commonly used (Fallu 1991).

Table 1.5 Comparison of spawning induction methods used for *Haliotis* species in hatcheries (Fleming 2000).

Species	**Induction method**
H. discus hannai	Temperature increase to 27°C for 1 h ⇨ expose to air for 1 h ⇨ UV ⇨ dark
H. iris	15°C ⇨ H_2O_2 ⇨ leave 3 h ⇨ flush ⇨ cover tanks
H. midae	H_2O_2 ⇨ leave 1 h ⇨ flush ⇨ keep in dark
H. rubra	UV ⇨ Temperature shock
H. rufescens	H_2O_2

Previous studies reported that abalones would start to spawn three to four hours after ultra-violet (UV) light was used to sterilise tank water. The reason for this is that when UV light reaches water, water molecules are split, releasing hydrogen peroxide (H_2O_2), which causes abalone to spawn (Morse *et al.* 1977). The use of UV light to induce spawning is a fast and reliable method but depends on two factors; i.e. the quality of the water and the amount of UV irradiation. Ultra-violet light will be deflected and dispersed by particles in seawater, reducing the actual amount of irradiation. For this reason, seawater needs to be filtered before irradiation, which could be costly. The optimal amount of UV irradiation should also be determined for each respective species, but the recommended amount is 2.42 W L^{-1} (Hahn 1988; Fallu 1992).

In 1977, the effect of hydrogen peroxide itself was tested and found to induce spawning in various abalone species. Currently, this is the most common method used to induce spawning in hatcheries due to the fast and inexpensive nature of the procedure. It is recommended that the concentration of H_2O_2 should be kept at 25 mg kg^{-1} but this dosage needs to be verified for each species, to ensure it is not too high or too low (Morse *et al.* 1977; Hahn 1988; Fallu 1991).

Broodstock animals on South African farms are spawned at six week intervals, using sodium hydroxide as an initial step to change water pH, after which hydrogen peroxide is added to tanks 15 minutes later. Three hours after the addition of hydrogen peroxide, tanks are emptied and washed out to rid them of any residual hydrogen peroxide, which could be toxic to gametes (Morse *et al.* 1977).

After this, the tanks are filled with fresh FSW with constant airflow. As soon as the tanks are filled, water flow is switched off to prevent dilution of gametes when spawning commences.

Males start spawning four hours after induction, at which stage a fixed volume of the densest sperm from random tanks (water with a milky appearance) is added to female tank. It has been shown that broadcast spawners can be synchronised to spawn by other spawning animals, arguing that the gametes released triggers mass spawning. For this reason, it is thought that adding sperm to female tanks will induce the females to spawn, although females will usually start spawning six hours post-induction, regardless of the addition of sperm. A limitation of this method is that sperm concentrations and time of fertilisation cannot be controlled, as eggs will be fertilised as they are released.

As soon as the females start to spawn, airflow is switched off for approximately 30 minutes to allow eggs to settle. Eggs are then siphoned using plastic tubing by gradient incline into 25 L plastic buckets passing through a 200 µm mesh to trap any debris in the egg masses. Subsamples are taken to evaluate fertilisation rate after which the fertilised eggs are transferred to hatch-out tanks. When evaluating the effectiveness of spawning inductions in various *Haliotis* species (**Table 1.6**), great variation in induction response is also observed. Spawning success on South African abalone farms are estimated at 25%, with some animals not contributing to spawnings at all.

Table 1.6 Spawning induction responses of various *Haliotis* species (Morse *et al.* 1977; Hahn 1988; Fleming 2000).

Species	**Spawning frequency**	**Mean spawning success (%)**		**Mean response time**	
		Male	**Female**	**Male**	**Female**
H. corrugata		60	15	2h30	3h00
H. discus	11 weeks	100	80	5h30	6h45
H. fulgens	2 weeks	40	30	3h00	4h00
H. iris		48	40	1h30	2h00
H. laevigata		16.7	37.5	8h45	> 8h45
H. midae	6 weeks	25	25	4h00	6h00
H. rubra	4 weeks			6h15	6h30
H. rufescens	12 weeks	80	80	2h30	3h00
H. sorenseni		100	15	2h00	2h30

1.6.4. Hatching

Twelve hours post-fertilisation, hatch-out occurs (**Figure 1.11**). At 24 hours post-fertilisation, veliger larvae will be transferred to larval rearing tanks, while underdeveloped or abnormal larvae will be flushed out of the hatch-out tanks (**Figure 1.12**).

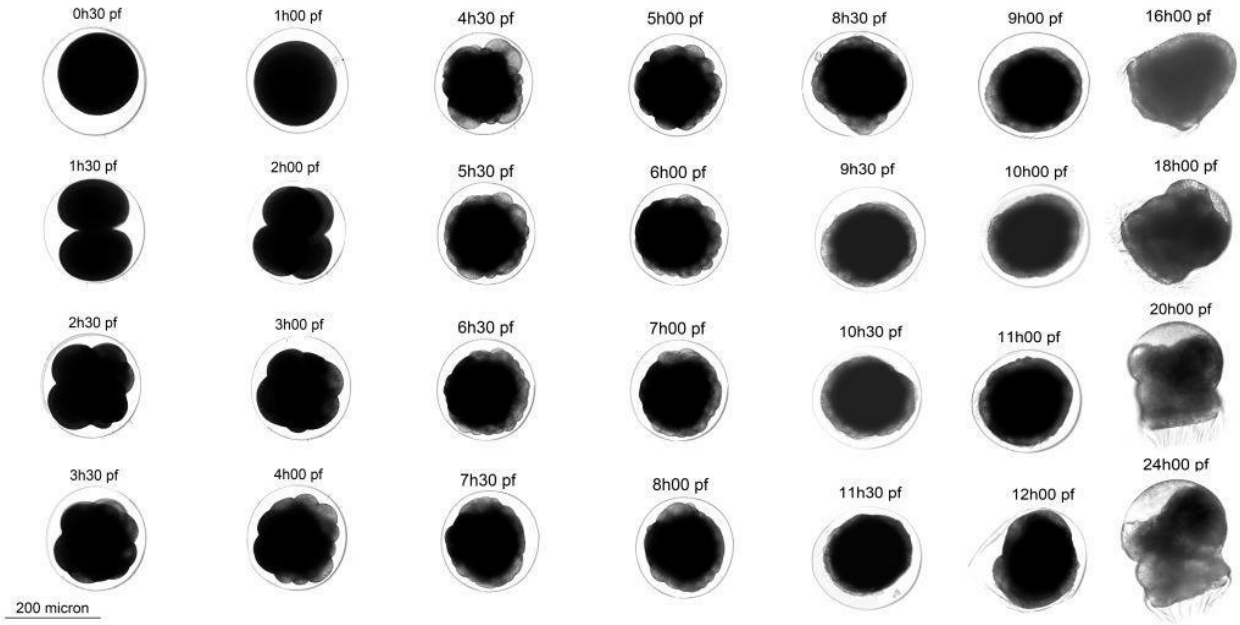

Figure 1.11 Developmental stages observed in the present study of *Haliotis midae* at 30 minute intervals post-fertilisation (pf).

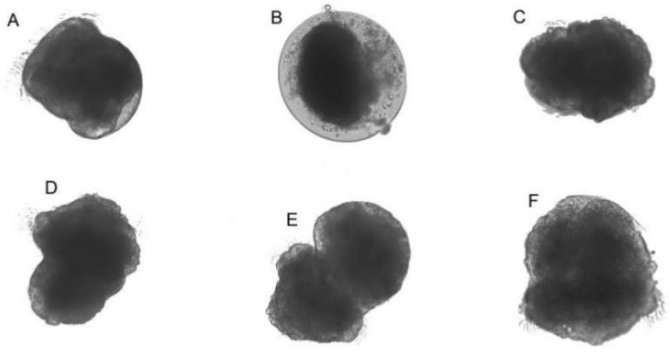

Figure 1.12 Various forms of *Haliotis midae* larvae. A = normal larvae; B = larvae arrested in development; C – F = morphological abnormalities (present study).

1.7. Overview of methods

This study was conducted on two commercial abalone farms in Hermanus on the south-west coast of South Africa; HIK Abalone Farm (34°26'1.60"S and 19°13'14.06"E) and Aquafarm Development (34°26'3.76"S and 19°13'17.19"E). All experimental work was performed in a certified class 1 laboratory at HIK Abalone Farm. Both farms cover an area of 2.5 ha and consist of 1 500 production tanks that produce a biomass of 125 ton (4.8 million animals) annually.

Production animals are cultured in oyster net baskets suspended in concrete tanks. Seawater is supplied at a flow rate of 90 L h^{-1}. Water temperature varies according to season, with natural spawning occurring in April and September. The animals are fed an artificial diet specifically formulated for abalone (Abfeed, Marifeed South Africa) and grown to a market size of 70 to 120 mm shell length (SL; equating to three to four years of age), before being processed for export.

1.7.1. Experimental animals and husbandry

Wild-harvested broodstock of unknown age from both HIK Abalone Farm and Aquafarm Development was used in this study. The animals had an average shell length and weight of 20±5.0 cm and 1.5±0.5 kg, respectively. Broodstock males and females were kept in separate tanks at a stocking density of five animals per tank. For the purpose of the study, six randomly selected tanks (three female tanks and three male tanks) were used. The tanks were supplied with aeration and filtered (1 µm) FSW at a flow rate of 90 L h^{-1}. Animals were kept in isolated rooms at a holding temperature of 18°C with photoperiod set on 12 hours light and 12 hours dark. The tanks were cleaned every third day after which animals were supplied with kelp (*Ecklonia maxima* or *Laminaria pallida*, depending on availability).

1.7.2. Spawning induction and gamete collection

The standard spawning protocol on the participating farms in this study is to spawn animals every six weeks. Due to this, different broodstock were used every week to shorten the duration of the trial and to minimise individual variations.

Spawning was achieved by using sodium hydroxide as an initial step, after which hydrogen peroxide was added to tanks 15 minutes later (Morse *et al.* 1977). One hour after the addition of hydrogen peroxide, tanks were emptied and washed out to rid them of any residual hydrogen peroxide, as this could be toxic to gametes. The tanks were then filled with fresh FSW with constant aeration. As soon as the tanks were filled, water flow was switched off to prevent the dilution of gametes when spawning

commenced. Males started spawning four hours after induction with females spawning at six hours post-induction.

Two hours after the onset of spawning, sperm were collected by sampling 100 mL seawater from three random tanks, each containing five animals, using a Pyrex beaker (Lasec). This was done to minimise the effect of individual variation. Sperm from all the tanks were pooled in a 250 mL Erlenmeyer flask and sperm counts done according to Hahn (1989) using a Neubauer haemocytometer and inverted microscope (IX 51 Inverted Fluorescent Microscope, Olympus). After counts, sperm were held at 18°C in a refrigerated incubator (LM 570, MRC) until used for fertilisation.

Thirty minutes into the female spawning, aeration and water-flow to female tanks was turned off to allow the eggs to settle. Eggs were siphoned from three random female tanks, each containing five animals, using a serological pipette (Sigma), and transferred to glass Erlenmeyer flasks (Lasec). Eggs from all three tanks were pooled and egg concentrations determined volumetrically by counting the number of eggs in 1 µL using an inverted microscope. Egg counts were repeated six times to verify the average number of eggs per 1 µL.

1.8. Objectives of the study

Hatchery protocols used in South African abalone farms are effective but are not considered optimal. The intention of this work was to optimise these procedures in order to increase hatchery output and add value to the industry.

The main objectives of this thesis are to:

- Characterise gonad microstructure at various stages of gametogenesis in cultivated abalone (Chapter 2)
- Identify the onset of sexual maturity for cultivated abalone (Chapter 2)
- Develop a fertilisation protocol specifically for *Haliotis midae* (Chapter 3)
- Develop a stress test to evaluate the larval competence in abalone (Chapter 4)
- Investigate the potential of hybridisation between two South African abalone species (Chapter 5).

1.8.1. Rationale and Aim of Chapter 2: Reproductive histology of cultured Haliotis midae

1.8.1.1 Rationale

Potential broodstock animals are selected for conditioning at the age of three to four years (70 to 120 mm SL). The reason for this is that gender is indistinguishable at a younger age, prohibiting hatcheries to determine the number of males and females selected for conditioning. Identifying gender histologically could assist in overcoming this problem. It would be beneficial to identify prolific breeders prior to conditioning, as the animals can then be introduced into the conditioning protocol at an earlier age. Based on histological assessment of animals of various ages, an estimated age of maturation can be predicted and used as benchmark for broodstock selection. It would also be helpful to determine the stage of maturation of an individual by characterising gametogenesis.

1.8.1.2 Aim

Firstly, it is necessary to histologically characterise the various stages of gametogenesis. Secondly, animals of various age groups will be examined to determine the onset of maturation in cultivated animals. To ensure that animals that have not yet spawned are selected, collection of animals should occur outside of their natural spawning cycle. If animals did spawn before the collection of gonad tissue, normal gametogenesis could be obscured.

1.8.2. Rationale and Aim of Chapter 3: Development of an experimental fertilisation procedure for Haliotis midae

1.8.2.1 Rationale

Currently, gamete concentrations are not measured in hatcheries before fertilisation. The implication of this randomised fertilisation protocol is that there is no control over sperm to egg ratios. This might lead to ineffective fertilisation due to a lowered gamete ratio or polyspermia where too high concentration of sperm is added to the eggs. Determining optimal gamete concentrations specifically for *Haliotis midae* before fertilisation might increase hatchery output.

Fertilisation and hatch-out success could be further complicated by the practise of adding sperm to female tanks before females start to spawn. This may result in asynchronous larval development. By fertilising all eggs simultaneously after collection and density determination, synchronised larval development can be achieved, ensuring that all larvae will hatch concurrently with no underdeveloped larvae being discarded. When the proposed fertilisation protocol is followed, where sperm and eggs are first collected and then counted, the aging effect of the gametes should be taken into

consideration. Eggs will lose viability if not fertilised within a certain period after spawning. To determine this window-period, it is essential to evaluate the loss of egg viability over time.

Whilst fertilisation volume does not appear to have a great effect on fertilisation success, it is important to consider the volume of water in relation to the gamete ratio especially in artificial fertilisation procedures. If the water volume is too large, fertilisation success might decrease even though optimal gamete ratios are used. This could be due to sperm losing motility in large volumes, decreasing the possibility of gamete interaction.

1.8.2.2 Aim

This part of the study aims to develop an optimised fertilisation protocol specifically for *Haliotis midae*, which could assist in synchronised larval development in hatcheries. This will be done by determining the optimal gamete ratios for successful fertilisation in specific volumes as well as the optimal time for fertilisation after spawning. Due to the long period between individual spawnings and the fact that not all animals will contribute to a spawning, various animals will be used in these experiments. Gametes of randomly selected tanks, containing up to five animals per tank, will be pooled. From this, gamete concentrations will be determined and diluted to achieve the studied concentrations as well as to determine the aging effect on eggs.

1.8.3. Rationale and Aim of Chapter 4: Determination of larval competence in Haliots midae *by using a toxicity assay*

1.8.3.1 Rationale

Presently, there are no criteria to measure larval competence in abalone. By determining the level of resistance larvae show against the effect of certain stressors, a protocol can be established to determine larval competence. Such a protocol will assist in identifying less viable larvae that can be eliminated from the system before grow-out. A larval competences test could also assist hatcheries in evaluating the output of individual broodstock. This will ensure that poor breeders can be identified and removed from the breeding program.

1.8.3.2 Aim

A toxicity assay will be developed that could assist hatcheries to determine the resistance of larvae to a given toxicant. By establishing the effective concentration of 50% (EC50) of the toxicant, fertilised eggs can be subjected to this and abnormalities measured after hatching. The results will give an indication of the ability of the larvae to resist toxic stress.

1.8.4. Rational and Aim of Chapter 5: Hybridisation of Haliotis midae *and* H. spadicea

1.8.4.1 Rationale

The preliminary evaluation of the hybridisation potential between *H. spadicea* and *H. midae* could lay the foundation for future experiments in determining the effect of heterosis on these species. Another potential advantage of hybrid production could be induced sterility, as little to no investment is made into gonad development. Since *H. spadicea* is not a cultured species, these animals cannot be chemically induced to spawn. This problem could be overcome by developing a biopsy method to collect gametes. It is however crucial that collection is non-destructive, as culling animals for gonad retrieval could eliminate possible valuable animals.

1.8.4.2 Aim

The aims of this study are firstly to determine the fertilisation potential of biopsied spermatozoa from *H. midae* and to establish the effect of repetitive biopsies on the survival of *H. midae* males. The second aim is to produce viable hybrid larvae using biopsied spermatozoa from *H. spadicea* and spawned eggs from *H. midae* and genetic verification of resultant hybrid larvae.

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Chapter 2 Reproductive histology of cultured *Haliotis midae*

2.1. Abstract

This study reports the first classification of cytological structures in developing gonads of cultivated *Haliotis midae*, as well as the confirmation of the age at onset of maturity. Gonad tissue from animals ranging from six months to 48 months of age was histologically evaluated. The process of oogenesis was divided into nine distinct stages according to the size and histological structures of each cell type, while spermatogenesis was divided into five distinct stages. Six month old animals showed initial development of germinal epithelium, while at 12 months of age the gonad cavity was visible. In males of 18 months of age, spermatogonia and primary spermatocytes were identified, while in 24 month old males, all cell stages of spermatogenesis were present, indicating animals to be sexually mature at this age. Oogonia and stage 1 oocytes were seen in the gonad cavity of an 18 month old female, while in the gonad of two year old females all stages of oogenesis could be distinguished, though mature oocytes were not abundant. Results of this study indicate that maturity in cultivated *H. midae* is reached by the age of 24 months, implying that animals of this age can be conditioned for spawning.

2.2. Introduction

Studies on gametogenesis abalone are numerous, and have mainly focused on spawning season, fecundity, and the effect of temperature on gonad development (Hahn 1994; Sobhon *et al.* 1999; Litaay & De Silva 2003; Grubert & Ritar 2004). Ultrastructural studies of spermatogenesis and oogenesis of *Haliotis ovina* and *H. varia* provided classification systems for the cytological structures of maturing germ cells in abalone by using light microscopy as well as transmission electron microscopy (TEM) (Singhakaew *et al.* 2003; Najmudeen 2008). Awaji and Hamano (2004) conducted the first study on gonad formation and sex differentiation processes during the juvenile stages of cultivated *H. discus hannai* to determine the onset of maturity in this species.

Gonad structure and development has been histologically described for wild-caught *H. midae*. It was estimated that the animals acquired full sexual maturity at a shell length of 110±10 mm and live weight of 350±50 g, which correlated to approximately seven years of age (Newman 1967). A later study found that sexual maturity could be achieved earlier, especially in animals found along the warmer eastern coast of South Africa (Wood & Buxton 1996). These authors concluded that onset of gametogenesis could be observed in animals with shell widths ranging from 20 to 25 mm (i.e. one year of age), with 50% maturity attained at a size of 35±2 mm; i.e. at approximately 24 to 36 months of age.

To date, no histological studies have been done on cultivated *H. midae*. The aim of this study was to classify cytological structures in developing gonads of animals ranging from six to 48 months of age. Histological evaluation of gonad tissue may potentially assist hatchery managers to determine the onset of maturity of cultivated *H. midae*, as well as to select broodstock based on early attainment of sexual maturity as a trait.

2.3. Materials and Methods

2.3.1. Experimental design

Experimental animals of known ages (from six to 48 months old at six monthly intervals; **Table 2.1**) were provided by HIK Abalone Farm and cultivated as described in Section 1.7. *Haliotis midae* naturally spawn during autumn (April) and spring (September). For this reason animals were selected during August 2010. During this period it could be assumed that gonad development would have been initiated but that animals had not yet spawned naturally. If animals did spawn before the collection of gonad tissue, normal gametogenesis could be obscured. Three males and three females were collected for each age category and shell length and net weight were recorded.

Table 2.1 Details of cultured *Haliotis midae* used in this study according to approximate age and average weight and size of animals.

Settlement date	Animal age (months)	Shucked weight (g)	Shell length (mm)	Shell width (mm)
Feb 2010	6	0.5	20.0	10.0
Aug 2009	12	2.0	30.0	20.0
March 2009	18	5.0	40.0	25.0
Aug 2008	24	10.0	50.0	30.0
March 2008	30	15.0	55.0	35.0
June 2007	36	45.0	70.0	45.0
Nov 2007	42	60.0	80.0	55.0
June 2006	48	175.0	120.0	85.0

After collection, the animals were euthanized by immersion in seawater containing 5% (w/v) magnesium chloride for one hour (Sobhon *et al.* 1999). Gonads from the age groups 24 to 48 months were dissected and preserved in 10% neutral buffered formalin (Sigma, South Africa), while animals from the age groups six to 18 months were preserved whole in 10% formalin. This was done as the gonads of these animals were too small to be dissected (**Figure 2.1**).

Figure 2.1 Size and age of *Haliotis midae* animals used in this study. 1 = six months; 2 = 12 months; 3 = 18 months; 4 = 24 months; 5 = 30 months; 6 = 36 months; 7 = 42 months; 8 = 48 months.

2.3.2. Histological preparation

Processing, sectioning, and staining of tissue as well as slide preparations were done by the Division of Anatomy and Histology of Stellenbosch University (Tygerberg, South Africa). Fixed samples were dehydrated in a series dilution of ethanol (70% to 100% v/v) for 180 minutes each, cleared with xylene for 150 minutes, and then embedded in paraffin wax for 120 minutes at 60°C. Sections of 5 µM were cut using a microtome (RM2265, Leica), and incubated at 60°C for 60 minutes in an autoclave.

The sections were rehydrated in a series dilution of ethanol (100% to 70% v/v), after which the sections were stained with haematoxylin for four minutes and eosin for 2.5 seconds (HE staining). After staining, samples were first dehydrated in a series dilution of ethanol (70 to 100% v/v), then cleared with xylene for one minute, and subsequently mounted on microscope slides (25 mm X 75 mm X 1 mm) with di-n-butyl phthalate in xylene (DPX) mounting medium (Fluka), and covered with a cover slide.

Slides were analysed using an inverted microscope (IX 51, Olympus), and micrographs were taken with an XC 10 Olympus camera. Classification of cell types was done according to the system followed in the study of Sobhon *et al.* (1999).

2.4. Results

2.4.1 Oogenesis

2.4.1.1 Oogonia

Oogonia were observed as round or ovoid cells, and ranged from 15 to 20 µm in diameter (**Figure 2.2 A**). Oogonia displayed round nuclei of about 10 µm, which stained light to dark blue with haematoxylin-eosin, as well as a strong basophilic nucleolus with an average size of 4 µm. The cytoplasm stained a dark blue, indicating the presence of a moderate amount of ribosomes. Oogonia occurred in groups attached to the trabeculae, and were usually surrounded by squamous-shaped follicular cells.

2.4.1.2 Stage 1 Oocytes

Stage 1 oocytes appeared more oval-shaped than the oogonia but still closely adhered to the trabeculae, and ranged from 20 to 25 µm in size (**Figure 2.2 B**). The oval nucleus was approximately 15 µm in size, and stained light blue with densely packed chromatin being visible. The nucleolus increased from 4 µm to 6 µm in size and remained strongly basophilic. The cytoplasm of stage 1 oocytes stained dark blue.

2.4.1.3 Stage 2 Oocytes

Stage 2 oocytes had lengths of between 30 to 35 µm, and exhibited a very lightly stained nucleus of approximately 20 µm in size in which uncoiling chromatin was visible (**Figure 2.2 C**). The nucleolus of stage 2 oocytes did not differ in size from that of stage 1 oocytes, and stained dark blue. The cytoplasm stained dark blue as well, with small lipid droplets observed in the cytoplasm. Stage 2 oocytes were arranged more loosely around the trabeculae.

2.4.1.4 Stage 3 Oocytes

Stage 3 oocytes increased in size to between 45 to 55 µm, and developed into scallop-shaped cells (**Figure 2.2 D**). The enlarged nucleus (25 µm) appeared clear, with uncondensed chromatin staining light blue. At this stage the nucleolus was approximately 8 µm in size, and still assumed a dark blue colour although it was a lighter shade than that of stage 2 oocyte nuclei. The cytoplasm showed a greater number of clear lipid droplets and spaces between the cells increased due to the formation of the chorion.

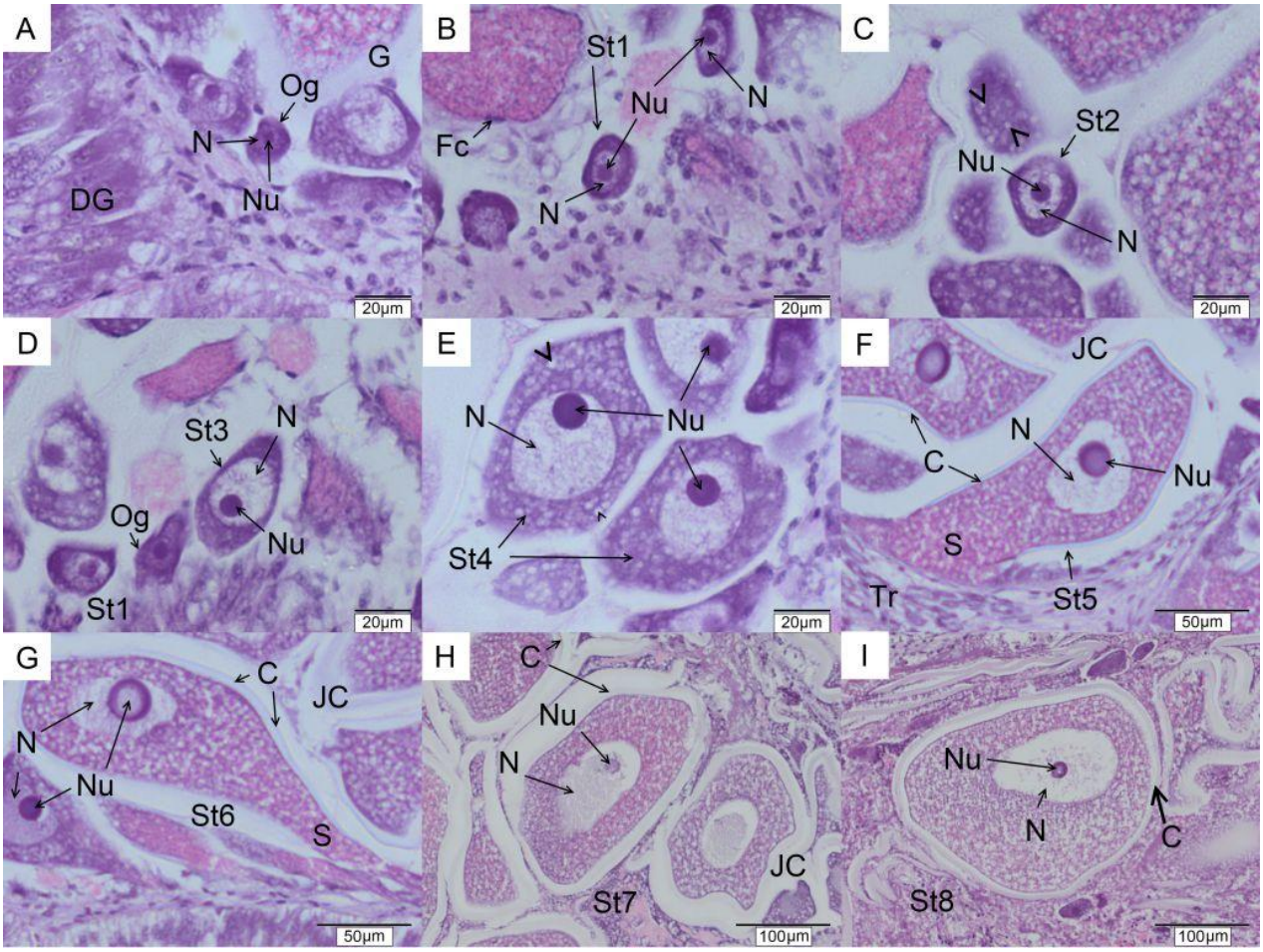

Figure 2.2 Oogenesis in *Haliotis midae*. C = chorion; DG = digestive gland; FC = follicular cell; GEp = gonad epithelium; JC = jelly coat; N = nucleus; Nu = nucleolus; Og = oogonia; S = cytoplasmic stalk; St1 = stage 1 oocyte; St2 = stage 2 oocyte; St3 = stage 3 oocyte; St4 = stage 4 oocyte; St5 = stage 5 oocyte; St6 = stage 6 oocyte; St7 = stage 7 oocyte; St8 = stage 8 oocyte; > = lipid droplets.

2.4.1.5 Stage 4 Oocytes

Lipid droplets in the cytoplasm of these cells increased in size, and appeared as a prominent feature, showing up as clear spots in the dark staining cytoplasm (**Figure 2.2 E**). Stage 4 oocytes ranged from 60 to 80 µm in size, with nuclear sizes of 35 to 45 µm. The nuclei contained unravelling chromatin, which stained light blue. The nucleolus ranged in size from 10 to 15 µm, and stained a lighter blue than that of the stage 3 oocytes. The chorion was more visible around each cell. Cells were now only attached to the connective tissue of the trabeculae at the base of the cell.

2.4.1.6 Stage 5 Oocytes

Stage 5 oocytes assumed a columnar shape, and ranged from 100 to 150 µm in length. The chorion and the attachment to the connective tissue of the trabeculae was clearly visible (**Figure 2.2 F**). The cytoplasm contained an increasing number of lipid droplets, and began to show yolk platelets, which resulted in the cytoplasm staining pink. The nucleus increased to 50 µm in size, and appeared almost completely transparent, with the euchromatin staining very light blue. The nucleolus became progressively lighter due to the uncoiling of the nucleolar chromatin, and varied in size from 20 to 25 µm.

2.4.1.7 Stage 6 Oocytes

These teardrop-shaped cells were attached to the connective tissue of the trabeculae by a very thin cytoplasmic stalk. In **Figure 2.2 (G)**, this stalk can be seen tearing loose from the connective tissue to form the next stage of oocytes. The clear chorion became encased by a thick jelly coat layer, which separated cells into loose cell clusters. The stage 6 oocytes reached lengths of up to 250 µm with the cytoplasm staining pink due to yolk platelets, and exhibited lipid droplets up to 4 µm in size. The nucleus developed into an ovoid structure and measured 50 µm, presenting a similar staining pattern to that of the stage 5 oocytes. The nucleolus became enlarged due to the uncondensed nucleolar chromatin, and had a nearly clear appearance, with a size of approximately 30 µm.

2.4.1.8 Stage 7 Oocytes

The nuclei of stage 7 oocytes were oval-shaped, with lengths of 100 µm, and contained mostly light blue stained euchromatin (**Figure 2.2 H**). The cytoplasmic stalk appeared completely separated from the connective tissue of the trabeculae, and the cells were now free in the lumen of the gonad, and covered by the chorion with a thick jelly coat surrounding them.

2.4.1.9 Stage 8 Oocytes

At this stage, the oocyte is mature and ready to be spawned (**Figure 2.2 I**). Sizes varied from 250 to 300 µm although in gravid gonads size was difficult to measure due to the compactness of the cells, which can distort their shape. The nucleus was completely clear, and ranged from 100 to 150 µm in size. The nucleolar chromatin of the nucleolus was completely unravelled. Lipid droplets in the cytoplasm were found in large clusters almost completely covering the cytoplasm, giving it a clear appearance, although the presence of yolk platelets resulted in the cytoplasm staining pink. The chorion and jelly coat became thinner as cells became more compressed.

2.4.2 Spermatogenesis

2.4.2.1 Spermatogonia

The spermatogonium is a large oval-shaped structure ranging from 5 to 7 µm in diameter, and contained a clear nucleus with small areas that stained light blue due to uncoiled euchromatin (**Figure 2.3**). The cells were closely attached to the trabeculae, and formed clusters that spread out into the lumen of the gonad.

2.4.2.2 Primary spermatocytes

Primary spermatocytes had an average diameter of 3 to 5 µm, and exhibited a prominent nucleus of 2.5 µm, with a dark staining nucleolus of 0.5 µm (**Figure 2.3**). The nucleus contained increasing amounts of dense heterochromatin as the cells developed and progressed further into the lumen.

2.4.2.3 Secondary spermatocytes

As the primary spermatocytes developed, the heterochromatin increased in size and density in the nucleus and obscured the nucleolus, giving these cells the appearance of darkly stained oval shapes of about 5 µm in length. At this stage, the cells were still loosely attached to the trabeculae (**Figure 2.3**).

2.4.2.4 Spermatids

The spermatids stained increasingly darker than the secondary spermatocyte due to the complete condensation of the heterochromatin, which obscured the nucleus under normal light microscopy. The cells were still oval-shaped, but ranged from 2 to 3 µm in diameter, and could be found free from the trabeculae (**Figure 2.3**).

2.4.2.5 Spermatozoa

Spermatozoa were elongated dark staining cells of approximately 2.5 to 3.5 µm in length. The ellipsoid nucleus contained completely dense heterochromatin, and these cells occurred free in the lumen of the gonad (**Figure 2.3**).

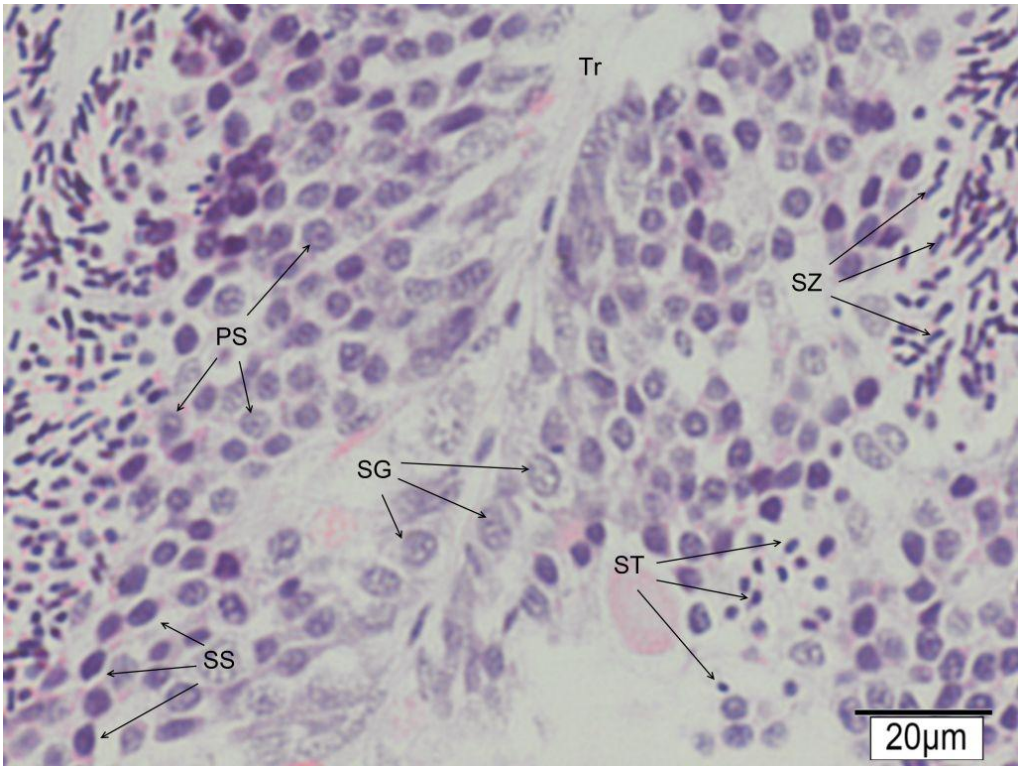

Figure 2.3 Spermatogenesis in *Haliotis midae*. PS = primary spermatocyte; SG = spermatogonia; SS = secondary spermatocyte; ST = spermatid; SZ = spermatozoa; Tr = trabeculae.

2.4.3 Comparison of gonad development between age categories

2.4.3.1 Six months vs. 12 months

Juveniles had an average shell width (SW) of 10 mm at six months of age, which increased to 20 mm in 12 months old animals. No gonad was visible by external evaluation, with the conical appendage containing both the digestive gland and the gonad tissue (**Figures 2.4 A** and **D**). Although primordial germ cells were visible, gender could not be established. Histologically, no germ cells were identified in six month old animals, although the germinal epithelium exhibited a degree of development at some areas of the conical appendage, forming a double cell layer with that of the digestive gland epithelium (**Figures 2.4 B** and **C**). The germinal epithelium measured approximately 3 µm in width, similar to that of the digestive gland epithelium. In 12 months old animals, a definite gonad cavity was observed (**Figure 2.4 E**), measuring from 15 to 30 µm in size, with progenitor germ cells developing from the epithelium into the gonad cavity. Squamous germ cells of 3 to 6 µm were visible along the germinal epithelium, but could not be identified (**Figure 2.4 F**).

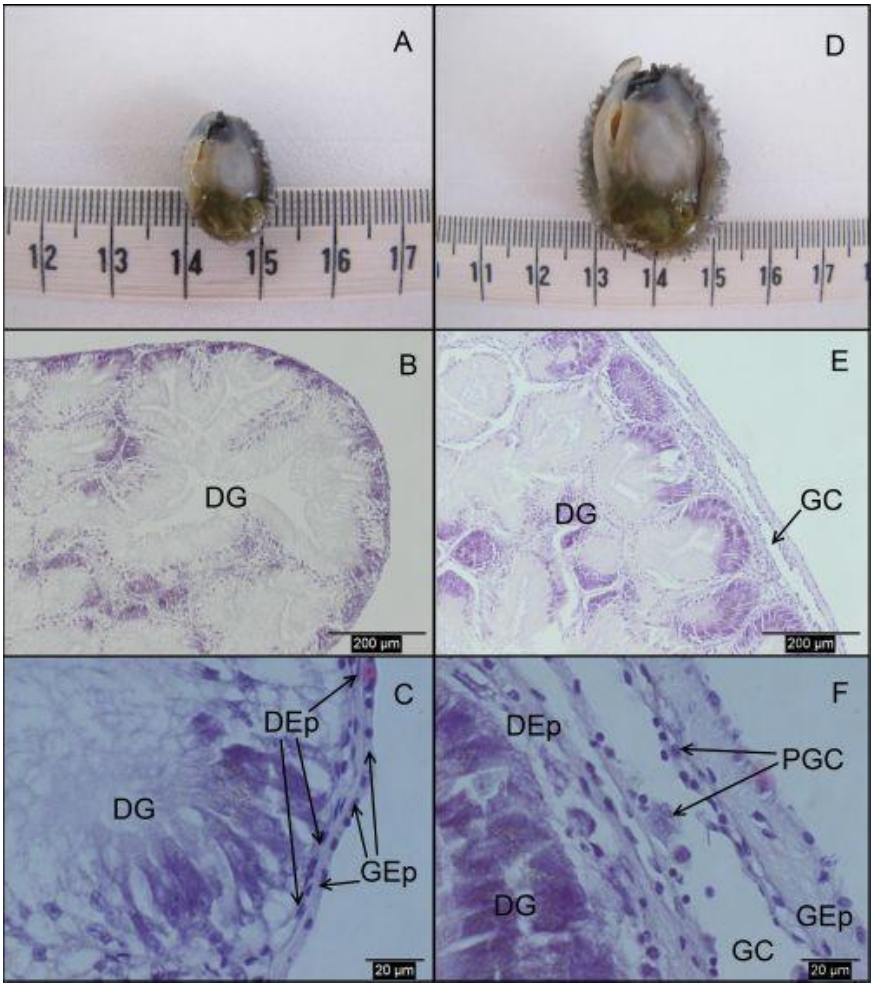

Figure 2.4 A comparison of the shell width (SW) of a six month old (A) vs. a 12 months old (D) *Haliotis midae* (gender not externally discernible) and gonad histology at six months (B & C) and at 12 months (E & F); DG = digestive gland; DEp = digestive gland epithelium; GEp = gonad epithelium; GC = gonad cavity; PGC = primordial germ cells.

2.4.3.2 Eighteen months vs. 24 months

Animals in the 18 month age group had an average SW of 25 mm increasing to 30 mm in the 24 month old age group. The gonad was not externally visible in these age groups, with the conical appendage having a uniform brown colour (**Figures 2.5 A** and **C**). In 18 month old animals, the gonad cavity was filled with gametogonia, and sexual differentiation between males and females was possible (**Figure 2.5 B**). Oogonia and stage 1 oocytes could be observed in the gonad cavity of an 18 month old female (**Figure 2.5 B**). In the gonad of a 24 month old female, all stages of oogenesis could be distinguished, with abundant stage 2 and 3 oocytes near the trabeculae (**Figure 2.5 D**). In 18

month old males, spermatogonia and primary spermatocytes could be identified, while in 24 month old males, all cell stages of spermatogenesis were present.

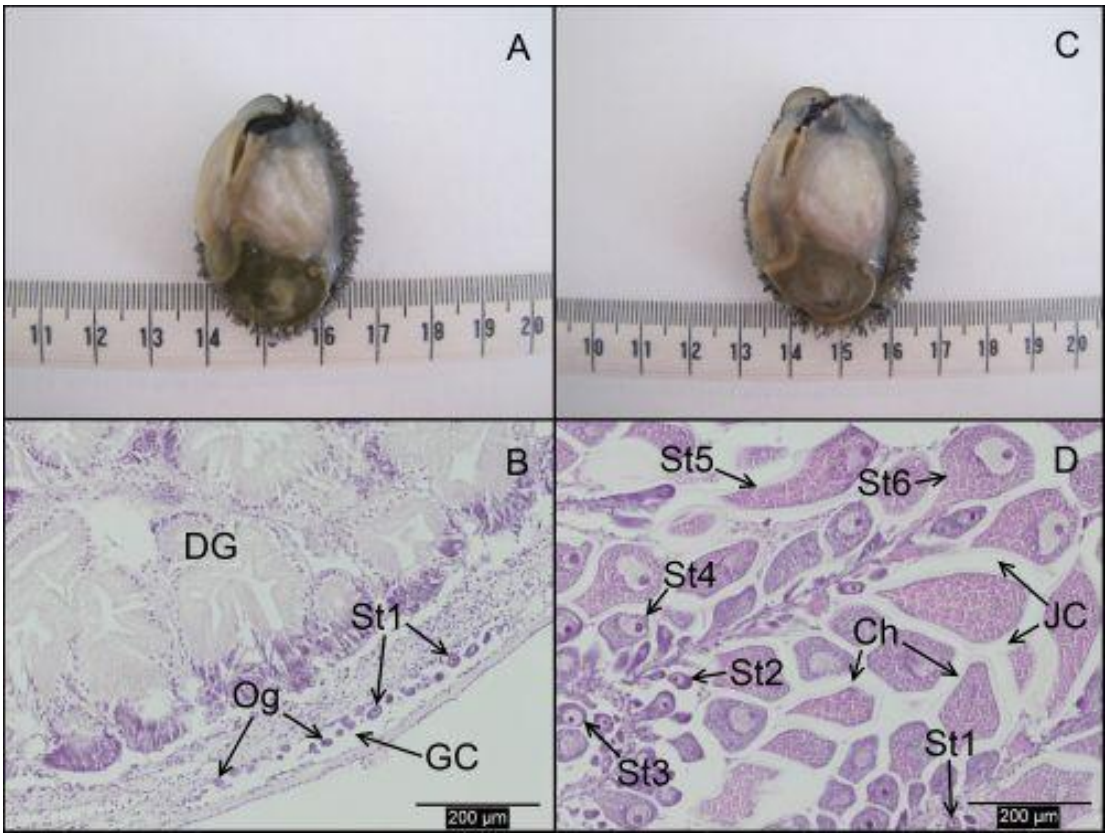

Figure 2.5 A comparison of the shell width (SW) of an 18 month old (A) vs. a 24 month old (C) *Haliotis midae* (gender not externally discernible) and gonad histology of a *H. midae* female at 18 (B) and 24 months (D) of age (gender histologically identifiable); Ch = chorion; DG = digestive gland; GC = gonad cavity; JC = jelly coat; Og = oogonia; St1, 2, 3, 4, 5, 6 = stage 1, 2, 3, 4, 5, 6 oocytes.

2.4.3.3 Thirty months vs. 36 months

At 30 months of age (i.e. 35 mm shell width), gonads remained indistinguishable from the digestive gland, with both organs exhibiting the same brownish colour (**Figure 2.6 A**). As the animals increased in size, gonads were more distinguishable from the digestive gland as colour appeared due to the presence of gametes. In 36 month old animals (i.e. 45 mm shell width) gonads were either white or green in colour, with spermatozoa contributing the white coloured gonad of the male and the green eggs causing the colour of the female gonad (**Figure 2.6 C**). Histologically, 30 month old females showed more oogonia and stage 1 to 3 oocytes than that of the 36 month old animals, in which larger stage 8 oocytes were more predominant, indicating a readiness to spawn (**Figures 2.6 B** and **D**). More oogonia and stage 1 to 3 oocytes were observed in the 30 month old females, when compared

to the 36 month old animals, in which the stage 8 oocytes were more predominant. The latter phenomenon indicated the readiness of the females to spawn.

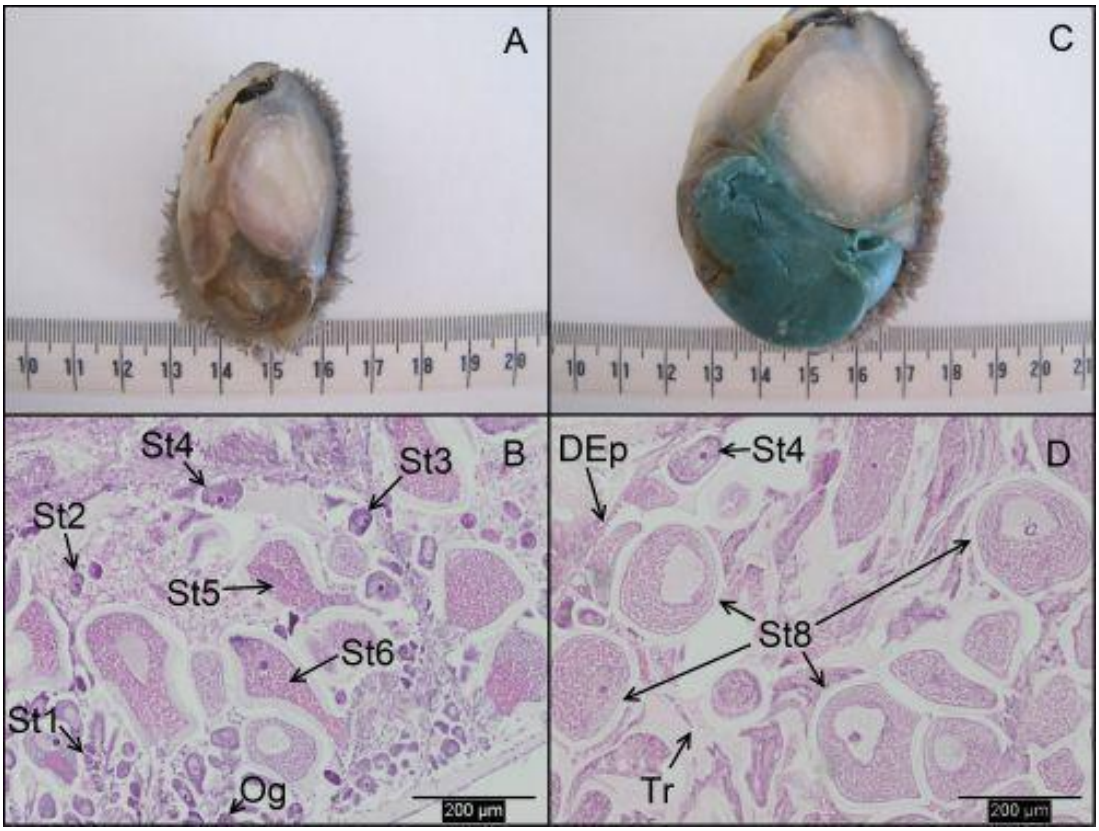

Figure 2.6 A comparison of the shell width (SW) of a 30 month old (A - gender not externally discernible) vs. a 36 month old *Haliotis midae* female (C - gender externally identifiable) and gonad histology of a *H. midae* female at 30 months (B) and 36 months (D) of age; DEp = digestive gland epithelium; Og = oogonia; St1, 2, 3, 4, 5, 6, 8 = stage 1, 2, 3, 4, 5, 6, 8 oocytes; Tr = trabeculae.

2.4.3.4 Forty-two months vs. 48 months

Males and females in the 42 and 48 month age groups displayed sizes of 55 and 85 mm in shell width, respectively. Gender could be determined by the external inspection of the gonads, which appeared highly gravid and easily discernable from the digestive gland (**Figures 2.7 A** and **C**).

Both these age groups displayed a large number of stage 2 and 3 oocytes, with stage 7 and 8 oocytes being more prominent than in the younger age groups (**Figures 2.7 B** and **D**). Jelly coats surrounding stage 7 and 8 oocytes appeared thick, and separated the oocytes, even though these cells became compressed and distorted as the gonad increased in size (**Figure 2.7 D**).

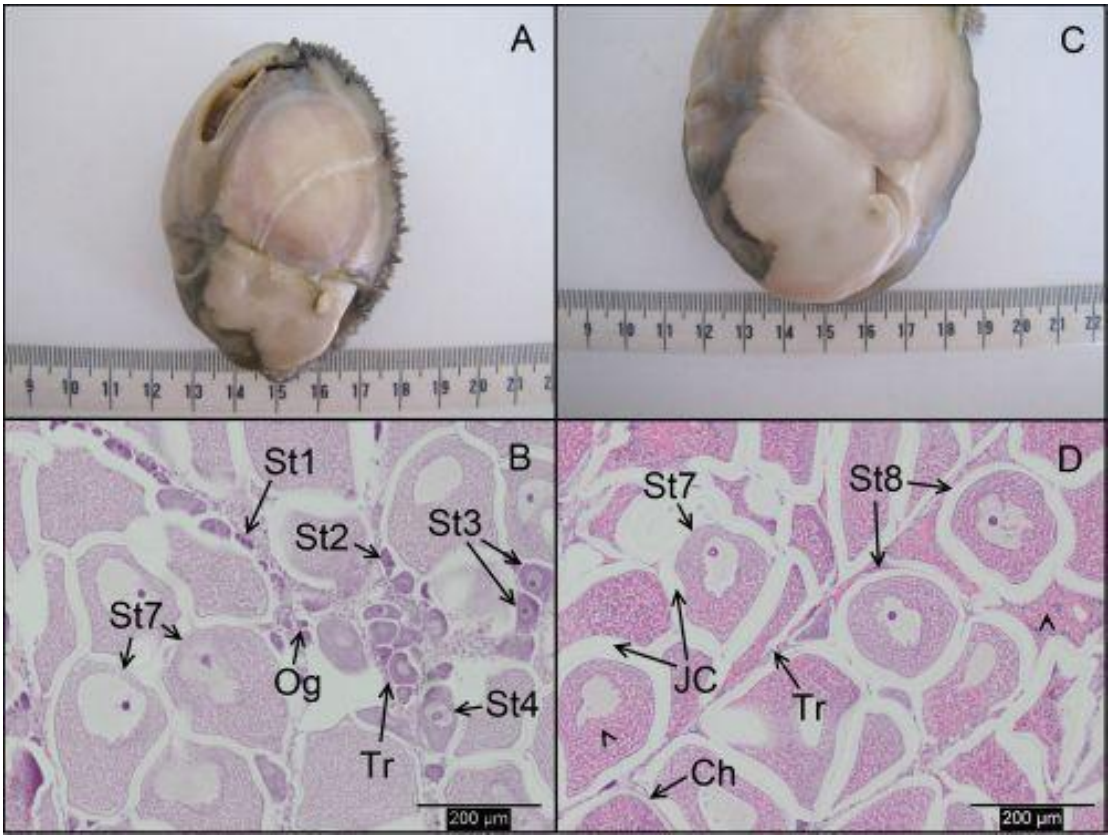

Figure 2.7 A comparison of the shell width (SW) of a 42 month old (A) vs. a 48 month old *Haliotis midae* male (C) and gonad histology of a *H. midae* female at 42 months (B) and 48 months (D) of age; Ch = chorion; JC = jelly coat; Og = oogonia; St1, 2, 3, 4, 7, 8 = stage 1, 2, 3, 4, 7, 8 oocytes; Tr = trabeculae; > = lipid droplets.

2.5. Discussion

Gonad structure of *Haliotis midae* has been described by Newman (1967) and Wood and Buxton (1996). These authors broadly characterised cell types based on cell sizes and not on specific histological features. According to Sobhon *et al.* (1999), constructing cell classification on size alone is not a reliable benchmark as these cells are constantly dividing and differentiating into various stages, thus undergoing continuous development. Describing histological structures as criterion for cell type classification provides a more standardised system to identify cells in the gonads, but due to variation in author criteria of different cell stages, it is difficult to compare gonad histology between abalone species.

Spermatogenesis was divided into five stages according to cell sizes and structure in this study (**Table 2.2**). This differed from previous studies were spermatogenesis was only divided into four cell stages based on cell sizes (Newman 1967; Wood & Buxton 1996).

Table 2.2 A histological comparison of cell types distinguished during spermatogenesis in *Haliotis midae* in this study compared to observations during previous studies.

	Newman (1967)	Wood and Buxton (1996)	Present study
Cell types	**Cell measurements (µm)**		
Spermatogonia	4.0	3.0	6.0
Spermatocytes	4.0	1.5	
Primary spermatocytes			4.0
Secondary spermatocytes			5.0
Spermatids	2.0	0.88	2.5
Spermatozoa	6.0	6.0	3.0

Spermatogonia examined in the present study exhibited a clear nucleus with small areas staining light blue due to uncoiled euchromatin. Primary spermatocytes were categorised separately from secondary spermatocytes, with primary spermatocytes showing distinct nuclei of 2.5 µm and a dark staining nucleolus of 0.5 µm. These cells were also round in shape in contrast to that of the secondary spermatocytes, which were ovoid with a length of 5 µm. In these cells, the heterochromatin obscured the nucleus, giving the cells the appearance of darkly stained ovals. Newman (1967) and Wood and Buxton (1996) classified primary and secondary spermatocytes in one category, reporting these cells to be 1.5 to 4 µm in size.

Spermatids were found to be of similar size to those described by Newman (1967), while Wood and Buxton (1996) found these cells to be on average 0.88 µm. The spermatids in the present study stained darker than the secondary spermatocytes due to the complete condensation of the heterochromatin, which obscured the nucleus under normal light microscopy. The spermatids were oval-shaped and ranged from 2 to 3 µm in diameter.

Spermatozoa were smaller than those described by Newman (1967) and Wood and Buxton (1996), both of which reported spermatozoa being on average 6 µm in size. In the present study, spermatozoa were 2.5 to 3.5 µm in length with an ellipsoid nucleus. No acrosome could be distinguished on spermatozoa in this study, although Wood and Buxton (1996) reported the presence of eosinophilic acrosomes. This might be because spermatozoa examined during the present study were not fully matured.

Newman (1967) divided oogenesis into three stages whereas four stages where identified by Wood and Buxton (1996). These authors described each stage according to cell size and orientation in the gonad. In the current study, oogenesis was divided into nine stages, based on the histological structures observed in each stage (**Table 2.3**).

Table 2.3 A comparison of cell types distinguished during oogenesis in *Haliotis midae* in the present and previous studies.

Cell types	Newman (1967)	Wood and Buxton (1996)	Present study
	Cell measurements (µm)		
Oogonia	10	14	15 – 20
Oocytes	50		
Previtellogenic oocytes		16 – 46	
Stage 1 oocytes			20 – 25
Stage 2 oocytes			30 – 35
Vitellogenic oocytes		50 – 145	
Stage 3 oocytes			45 – 55
Stage 4 oocytes			60 – 80
Stage 5 oocytes			100 – 150
Stage 6 oocytes			250
Mature oocytes	220 – 290	150 – 224	
Stage 7 oocytes			100
Stage 8 oocytes			250 – 300

In the present study, oogonia were 15 to 20 µm in size, slightly larger than the 14 µm reported by Wood and Buxton (1996). Newman (1967) reported oogonia to be 10 µm in size and further described “eggs on stalks” of 50 µm in size and eggs in the gonad lumen ranging from 220 to 290 µm. Wood and Buxton (1996) reported on previtellogenic oocytes of 16 to 46 µm with a basophilic cytoplasm. In the present study, these cells were divided into stage 1 to stage 2 oocytes. Stage 1 oocytes were 20 to 25 µm in size with the nucleus densely packed with heterochromatin. The nucleolus and cytoplasm stained strongly basophilic. Stage 2 oocytes increased in size to lengths of 30 to 35 µm and exhibited a very lightly stained nucleus in which the euchromatin was visible. The cytoplasm was still basophilic, although small clear lipid droplets became detectable. Vitellogenic stages were divided into stage 3 to stage 6 oocytes in the present study. Large vitellogenic cells of 50 to 145 µm attached to the trabeculae by cytoplasmic stalks were reported to emerge from the previtellogenic cells by Wood and Buxton (1996). These cells were described as having a basophilic nucleus and an increasingly eosinophilic cytoplasm as vitellogenesis began. Teardrop-shaped cells indicated the development of stage 6 oocytes, which were attached to the connective tissue of the trabeculae by a very thin cytoplasmic stalk. These cells reached lengths up to 250 µm with an eosinophilic cytoplasm containing a large nucleus and a prominent nucleolus in which euchromatin was visible. The cells in this stage was reported by Newman (1967) to be 50 µm, while free eggs in the lumen were reported to be between 220 to 290 µm in size by Newman (1967), and 150 to 224 µm by Wood and Buxton (1996). Although it is difficult to compare results from the present study with observations in

histological studies performed on other abalone species due to differences in author criteria, similarities in classification schemes were found. Work done on oogenesis in *H. asinina* (Apisawetakan *et al.* 2001) was comparable to the results described here (**Table 2.4**).

Table 2.4 A comparison of the classification of oogenesis, based on histological structures developed in the present study with observations during studies on *H. asinina* and *H. discus hannai* (Tomita 1967; Apisawetakan *et al.* 2001; Gurney & Mundy 2004).

Classification	***H. midae***	***H. discus hannai***	***H. asinina***
Oogonia: round to oval cell; small nucleus; dark-staining round nucleolus	Cell size: 15 – 20 µm Nucleus: 10 µm Nucleolus : 4 µm	Cell size: 4 µm	Cell size: 10 µm Nucleus: 7 µm
Stage 1 oocytes: oval-shaped; dense chromatin in nucleus; dark nucleolus	Cell size: 20 – 25 µm Nucleus: 15 µm Nucleolus: 6 µm	Not described	Cell size: 15 µm Nucleus: 12 µm
Stage 2 oocytes: oval-shaped; uncoiling nucleus chromatin; dark nucleolus	Cell size: 30 – 35 µm Nucleus: 20 µm Nucleolus: 6 µm	Cell size: 10 µm	Not described
Stage 3 oocytes: scallop-shaped; clear nucleus; oil droplets in cytoplasm	Cell size: 45 – 55 µm Nucleus: 25 µm Nucleolus: 8 µm	Cell size: 50 µm	Cell size: 55 µm Nucleus: 22 µm
Stage 4 oocytes: abundant lipid droplets; unravelling chromatin in nucleolus; chorion visible	Cell size: 60 – 80 µm Nucleus: 35 – 45 µm Nucleolus: 10 – 15 µm	Cell size: 70 µm	Not described
Stage 5 oocytes: columnar shaped; yolk platelets in cytoplasm; vacuoles in nucleolus	Cell size: 100 – 150 µm Nucleus: 50 µm Nucleolus: 20 – 25 µm	Cell size: 150 µm	Cell size: 70 µm Nucleus: 25 µm
Stage 6 oocytes: teardrop-shaped; attached by cytoplasmic stalk; jelly coat visible	Cell size: 250 µm Nucleus: 50 µm Nucleolus: 30 µm	Cell size: 170 µm	Cell size: 80 µm Nucleus: 35 µm
Stage 7 oocytes: oval-shaped; free in lumen	Cell size: not measured Nucleus: 100 µm	Not described	Not described
Stage 8 oocytes: mature cell	Cell size: 250 – 300 µm Nucleus: 100 – 150 µm	Cell size: 180 µm	Cell size: 140 µm Nucleus: 40 µm

The results of this study concur with the findings of Wood and Buxton (1996) in regard to the onset of sexual development in *H. midae*. Formation of the gonad cavity and thus onset of sexual development commenced at a shell width of 20±5 mm, and a shell length of 30 to 40 mm, relating to an age of 12 to 18 months, respectively.

In the present study, *H. midae* males were found to attain 50% sexual maturity at the age of 18 months (i.e. 25 mm shell width), while females attained 50% sexual maturity at 24 months of age (30 mm shell width). This is in contrast to findings from Wood and Buxton (1996) (**Table 2.5**), who reported *H. midae* to attain 50% sexual maturity at a shell width of 35 mm (i.e. 30 months of age). At 36 months of age (i.e. 50 mm shell width), gender can be determined by external examination, as both males and females possess large quantities of mature gametes at this stage, indicating a readiness to spawn.

Table 2.5 A comparison of results on the onset of sexual maturity in *Haliotis midae* in this study (expressed as mean percentage ± the standard error of the mean), with values reported in literature.

Age (years)	Shell width (mm)	Shell length (mm)	Present study	Wood and Buxton (1996)
0.5	10±3.0	17±2.0		
1.0	20±2.0	30±2.3	Gonad cavity formation	Gonad cavity formation
1.5	25±1.5	41±3.0	50% maturity in males	
2.0	30±1.0	48±3.0	100% maturity in males 50% maturity in females	
2.5	35±2.0	52±2.5		50% maturity in both genders
3.0	45±1.0	70±3.5	100% maturity in females Gonad colour visible	

2.6. Conclusion

This study indicates that commercially cultivated *H. midae* can reach 50% sexual maturity at 18 to 24 months of age. A constraint of this study was that animals of only one farm could be selected, reducing the application of this study on other abalone farms, although the techniques developed might be used in preliminary protocols for implementation on other farms. Another limitation was the

number of animals selected for this preliminary trial due to cost-implications and logistics. It should also be taken into consideration that water temperature might influence gonad maturation. In warmer regions, gonad maturation might be initiated earlier than in colder regions as observed between *H. midae* species occurring along the eastern coast of South Africa compared to that of the western coast (Wood & Buxton 1996). Despite these limitations, it could be concluded that broodstock conditioning may be initiated when animals in artificial systems reach an age of 24 months, as stage 6 and 7 oocytes are already developed at this age in females. This is 12 months earlier than the current practice of conditioning animals at the age of 36 months. Conditioning broodstock at an earlier age will enable hatchery managers to evaluate broodstock potential sooner and thus exclude individuals with poor reproductive output from the production system.

With the potential use of triploidy as a way of establishing sterility and increased growth in many commercial abalone species, including *H. midae*, (Arai *et al.* 1986; Yang *et al.* 1998; De Beer 2004) the results from this study can also be used as a tool to establish whether gonad and gamete development takes place in triploid animals and if so, to what extent.

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Chapter 3 Development of an experimental fertilisation protocol for *Haliotis midae*

3.1. Abstract

In this study, aspects of fertilisation such as gamete concentration and the time of fertilisation were evaluated and optimised specifically for *Haliotis midae*. Sperm concentrations between 5 000 and 50 000 sperm mL^{-1} did not influence fertilisation success, and consistently resulted in high hatch-out rates (96±1%). At sperm concentrations higher than 500 000 mL^{-1}, hatch-out rates declined to 69±7%, indicating that 50 000 sperm mL^{-1} is the optimal sperm concentration for fertilisation in *H. midae*. Egg concentrations between 10 to 125 eggs mL^{-1} resulted in high fertilisation rates, with 50 eggs mL^{-1} being the optimal concentration for fertilisation. The optimal time for fertilisation was 100 minutes post-spawning, with a decrease in hatch-out success when eggs were fertilised 120 minutes post-spawning. All fertilisation volumes used in this study resulted in high hatch-out rates, except when fertilisation was performed in 1 mL tissue culture wells. The results from this study may assist hatchery managers in South African abalone farms improve production efficiency and prevent or address problems such as low and variable fertilisation rates.

3.2. Introduction

Research on the development of abalone production in South Africa is mainly industry driven and is centred on the improvement of commercial systems, with emphasis on overcoming problems such as slow and variable growth. Research conducted during the last decade has focussed primarily on aspects such as nutrition, disease, ranching and genetics with little attention paid to reproductive strategies (Shipton & Britz 2001; Macey & Coyne 2005; Roodt-Wilding 2007; Roux *et al.* 2008; Slabbert *et al.* 2009). Research on the reproduction of *H. midae* is limited to ecological studies that reported on sex ratios and the onset of maturity in the wild (Newman 1967; Tarr 1989; Wood & Buxton 1996). Except for experimental hatchery methods studied in the 1980s, no additional scientific publications have emerged on the development of hatchery techniques for the commercial cultivation of *H. midae* (Genade *et al.* 1988).

Fertilisation protocols currently used on South African abalone farms are based on procedures developed for other abalone species, and may not be applicable to *H. midae* (Morse 1989; Fleming & Hone 1996). Hence, aspects of hatchery production on South African farms could be further optimised. Factors such as optimal gamete densities, time elapsed between gamete release and fertilisation, as well as fertilisation volumes have been found to affect fertilisation success in several sessile and sedentary molluscan broadcast spawners (O'Connor & Heasman 1995; Song *et al.* 2009).

The first aspect in the optimisation of fertilisation protocols is to determine the optimal sperm densities for fertilisation as this is known to vary between abalone species. It is thus necessary to determine the optimal sperm concentration for fertilisation for each species (Encena *et al.* 1998; Grubert *et al.* 2005). Fertilising eggs at optimal sperm concentrations maximise fertilisation rates, and avoid potential problems such as low and variable hatch-out success, as well as polyspermia (Hahn 1988; Encena *et al.* 1998).

Similarly, optimal egg concentrations for experimental fertilisation should be evaluated. Few studies on optimal egg densities have been conducted on molluscs (Crawford *et al.* 1986; Rampersad *et al.* 1994; O'Connor & Heasman 1995). In mangrove cupped oyster (*Crassostrea rhizophorae*), larval survival and egg concentrations were inversely proportional when a range of egg concentrations was used in fertilisation trials (Rampersad *et al.* 1994). In abalone, it is generally considered that a monolayer of eggs is sufficient for optimal fertilisation; however this has not been verified for *H. midae* (Hahn 1988).

Another factor affecting fertilisation success is egg viability. The decline in egg fertilisability is highly variable in abalone, with eggs of *H. gigantea* showing a significant decrease in viability in as little as five minutes post-spawning. Eggs of *H. corrugata*, *H. fulgens*, *H. laevigata*, *H. rufescens*, and *H. sorenseni* start losing viability at three hours post-spawning, while eggs of *H. diversicolor* and *H. tuberculata* loose viability within 30 to 60 minutes post-spawning respectively, and become non-viable at two and a half hours post-spawning (Leighton 1974; Babcock & Keesing 1999; Baker & Tyler 2001; Ahmed *et al.* 2008). By determining the optimal gamete age at fertilisation, fertilisation protocols could be improved thereby enhancing hatchery output (Song *et al.* 2009).

Although fertilisation volume is usually not monitored during hatchery procedures, it should also be considered a potential factor influencing fertilisation success. In large volumes, gamete densities may become compromised. Sperm could lose motility in larger volumes, which might result in the requirement of a higher sperm concentration for successful sperm-egg binding. It is thus important to determine the optimal sperm-egg ratios in a given volume (Song *et al.* 2009).

The aim of this study was to develop a small-scale fertilisation protocol specific to *H. midae* by determining optimal gamete concentrations, optimal time for fertilisation as well as optimal fertilisation volume. The results from this study could be used as a model for the improvement and optimisation of current hatchery procedures practised on South African abalone farms.

3.3. Materials and Methods

Experimental procedures followed here are described in Section 1.7. As the standard spawning protocol on farms participating in this study is to spawn animals every six weeks, different animals were used every week during this trial.

3.3.1. Determining the optimal sperm concentration for fertilisation

To determine the optimal sperm concentration for fertilisation, pooled sperm obtained from three tanks each containing five males, were diluted to various concentrations (i.e. $5x10^2$, $1x10^3$, $5x10^3$, $1x10^4$, $5x10^4$, $1x10^5$, $5x10^5$, $1x10^6$ and $1x10^7$ sperm mL^{-1}, respectively; hereafter referred to as sperm treatments) and used to fertilise 1 000 pooled eggs (Encena *et al.* 1998). Fertilisations were done in three Erlenmeyer flasks containing 200 mL FSW per sperm treatment. Fertilised eggs were then kept at 18°C in a refrigerated incubator (LM 570-R, MRC).

Twenty four hours post-fertilisation, hatch-out rates were determined microscopically, and recorded as the proportion of a) number of hatched larvae, b) number of abnormal larvae, and c) number of unfertilised eggs in relation to the total number of eggs used for fertilisation. Larvae were considered abnormal when they showed irregular shape, abnormal cilia, decreased size, arrested development (one cell stage to immature veliger), or if larvae were trapped in the egg membrane. The different types of abnormalities were grouped into a single category (Chapter 1; Figure 1.12).

3.3.2. Determining the optimal egg concentration for fertilisation

To determine the effect of egg concentration on fertilisation success, various egg concentrations (i.e. 10, 50, 125, 250, 375 and 500 eggs mL^{-1}, respectively; hereafter referred to as egg treatments) were tested in fertilisation experiments. Pooled egg batches from three tanks each containing five females, were used to achieve the test concentrations. Fertilisations were done in three 250 mL Erlenmeyer flasks containing 200 mL FSW per test concentration using the optimal sperm concentration of 50 000 sperm mL^{-1} as previously determined. Incubation methods and the assessment of hatch-out success followed that described in Section 3.3.1.

3.3.3. Determining the optimal time for fertilisation

The effect of ageing on eggs post-spawning was investigated by assessing hatch-out rates of eggs fertilised at twenty minute intervals (i.e. 20, 40, 60, 80, 100, 120, 140, 160 and 180 minutes post-spawning, respectively; hereafter referred to as time treatments) post-spawning. Pooled egg batches

from three randomly selected tanks each containing five females, were used. Fertilisations for each time interval took place in three 250 mL Erlenmeyer flasks containing 200 mL FSW per treatment using the optimal sperm concentration determined previously. Egg concentrations were kept at 10 eggs mL^{-1} for ease of handling. Incubation methods and the assessment of hatch-out success followed that described in Section 3.3.1.

3.3.4. Determining the optimal fertilisation volume

To determine the effect of various fertilisation volumes (i.e. 1 mL, 4 mL, 10 mL, 50 mL, 100 mL, and 200 mL; hereafter referred to as volume treatments) on hatch-out success, fertilisations were achieved using the optimal gamete ratios. Fertilisations were done in triplicate either in tissue culture chambers (Sigma) for smaller volumes (1 mL, 4 mL, and 10 mL FSW), or in 100 mL Pyrex beakers (Lasec) for larger volumes (50 mL, 100 mL, and 200 mL FSW). Incubation methods and the assessment of hatch-out success followed that described in Section 3.3.1

3.3.5. Statistical analysis

The data on hatching success for the optimal sperm, egg, time and volume experiments were pooled and expressed as percentages. To ensure a normal distribution of the data, all proportional data were arcsine transformed using Statistica 7 before a paired t-test was applied. Analysis of variance was used to determine significant differences between treatments. Results are presented as the mean percentages with standard errors. Each experiment was repeated over three consecutive spawnings to obtain up to nine data points per experiment.

3.4. Results

3.4.1. Optimal sperm concentration for fertilisation

Sperm concentrations ranging from 1 000 to 100 000 sperm mL^{-1} resulted in high fertilisation rates ($p > 0.05$) but were more consistent between 5 000 and 50 000 sperm mL^{-1}, with a sperm concentration of 50 000 sperm mL^{-1} yielded the highest fertilisation rate (96% hatch-out). Sperm concentrations lower than 1 000 mL^{-1} produced mean hatch-out rates of 52±3%, while at concentrations higher than 500 000 mL^{-1}, hatch-out rates averaged just 40±5% **(Table 3.1)**.

Table 3.1 Fertilisation and hatch-out success rates (mean±SEM) of *H. midae* larvae relative to sperm concentration at fertilisation.

Sperm concentration (sperm mL^{-1})	Mean normal larval hatch-out rate (%)	Mean abnormal larval hatch-out rate (%)	Mean unfertilised eggs rate (%)
$5x10^2$	$52±3.0^a$	2.0±0.0	50±4.0
$1x10^3$	$87±3.0^b$	6.0±2.0	20±7.0
$5x10^3$	$96±1.0^b$	3.0±0.0	1.0±0.0
$1x10^4$	$94±2.0^b$	8.0±3.0	1.0±0.0
$5x10^4$	$96±1.0^b$	3.0±1.0	1.0±1.0
$1x10^5$	$89±2.0^b$	11±2.0	1.0±0.0
$5x10^5$	$69±7.0^{ab}$	31±7.0	0.0±0.0
$1x10^6$	$42±7.0^a$	58±7.0	0.0±0.0
$1x10^7$	$11±1.0^c$	89±1.0	0.0±0.0

$^{a, b, c}$ Similar superscripts indicate no significant difference ($p > 0.05$).

Sperm concentrations of 500 sperm mL^{-1} resulted in a high levels ($p < 0.05$) of unfertilised eggs (50±4%), while an inverse relationship was observed at sperm concentrations higher than 500 000 sperm mL^{-1} which resulted in an increased ($p < 0.05$) occurrence (31±7%) of abnormal trochophores **(Figure 3.1)**.

3.4.2. Optimal egg concentration for fertilisation

Hatch-out rates did not differ ($p > 0.05$) between fertilisations when using 10 to 125 eggs mL^{-1} for fertilisation **(Table 3.2)**.

Table 3.2 Hatch-out rates (mean±SEM) of *H. midae* larvae relative to egg concentration at fertilisation.

Egg concentration (eggs mL^{-1})	Mean normal larval hatch-out rate (%)
10	$94±2.0^a$
50	$96±0.0^a$
125	$87±3.0^{ab}$
250	$77±6.0^b$
375	$39±7.0^c$
500	$15±2.0^d$

$^{a, b, c, d}$ Similar superscripts indicate no significant difference ($p > 0.05$).

An optimal hatch-out rate (96±0%) was achieved with an egg concentration of 50 eggs mL^{-1}, with hatch-out rates decreasing rapidly if egg concentrations higher than 250 eggs mL^{-1} were used for fertilisation.

3.4.3. Optimal time for fertilisation

Hatch-out rates were consistent when eggs were fertilised between 20 and 100 minutes post-spawning but started to decline at 120 minutes post-spawning **(Table 3.3)**.

Egg viability began to decline beyond 100 minutes post-spawning. There was no difference in the number or type of larval abnormalities between the different egg age treatments ($p > 0.05$). The optimal time range for fertilisation of *H. midae* eggs was as soon as possible after spawning up to 100 minutes post-spawning **(Figure 3.4)**.

Table 3.3 Fertilisation and hatch-out success rates (mean±SEM) of *H. midae* larvae relative to egg age at fertilisation.

Egg age (min)	Mean normal larval hatch-out rate (%)	Mean abnormal larval hatch-out rate (%)	Mean unfertilised eggs rate (%)
20	89±2.0[a]	8.0±1.0	3.0±1.0
40	87±3.0[a]	10±2.0	3.0±1.0
60	87±2.0[a]	8.0±1.0	5.0±1.0
80	83±3.0[a]	11±2.0	6.0±1.0
100	84±3.0[a]	7.0±1.0	9.0±2.0
120	65±8.0[ab]	8.0±1.0	27±8.0
140	61±10[ab]	5.0±1.0	34±10
160	52±11[b]	5.0±1.0	43±12
180	48±10[b]	7.0±1.0	45±9.0

[a, b] Similar superscripts indicate no significant difference ($p > 0.05$).

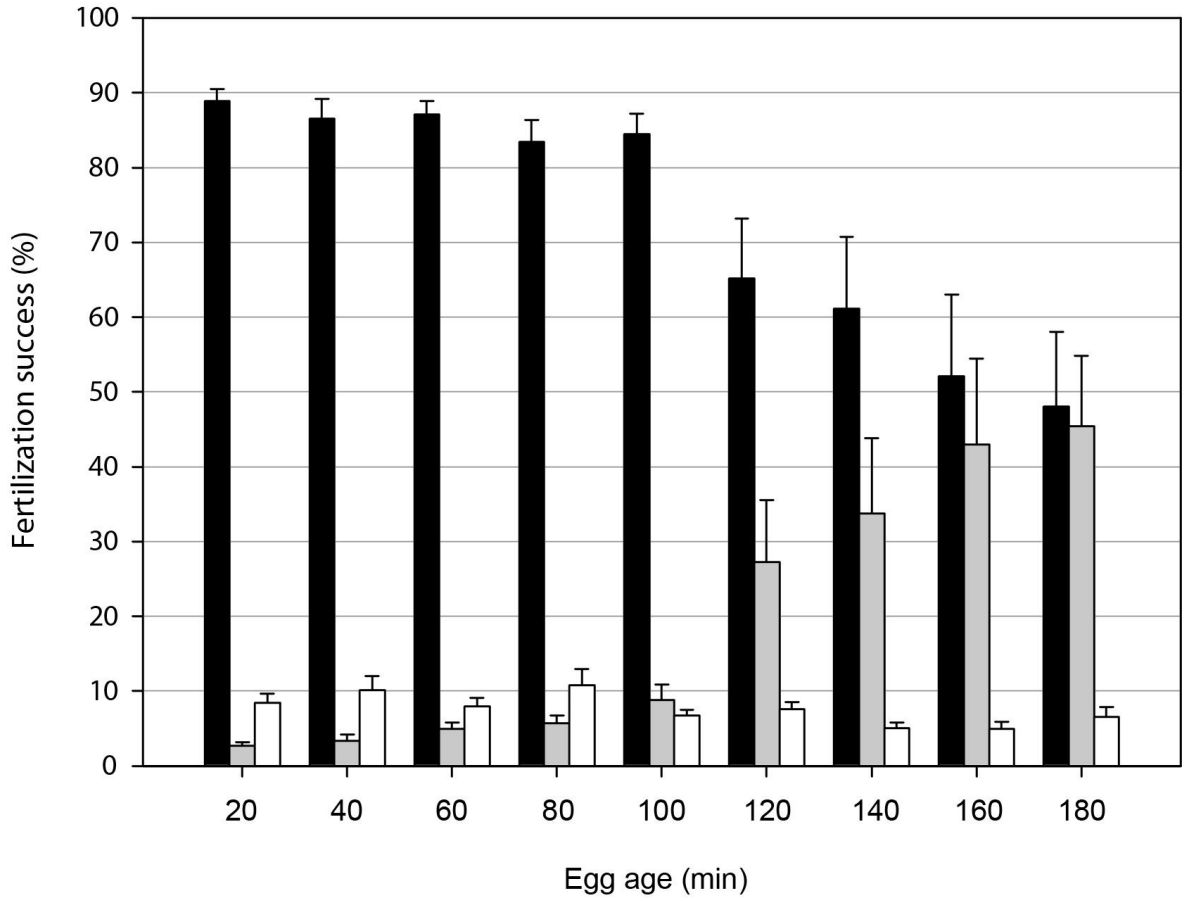

Figure 3.1 The influence of ageing on the fertilisability of *Haliotis midae* eggs, as assessed by means of fertilisation success (Unfertilised eggs = grey bars; Abnormal larvae = white bars; Normal larvae = black bars).

3.4.4. Optimal fertilisation volume

Fertilisations success at optimal gamete ratios was not affected by fertilisation volumes ($p > 0.05$), except at 1 mL FSW where fertilisation was negatively influenced ($p < 0.05$). Optimal fertilisation volumes ranged from 10 mL to 200 mL FSW, with fertilisations performed in 200 mL FSW producing the best results **(Table 3.4)**. Fertilisation volume did not have an influence on the occurrence of abnormal trochophores ($p > 0.05$).

Table 3.4 Fertilisation and hatch-out success rates (mean±SEM) of *H. midae* larvae relative to fertilisation volume.

Fertilisation volume (mL)	Mean normal larval hatch-out rate (%)	Mean abnormal larval hatch-out rate (%)	Mean unfertilised eggs rate (%)
1	20±4.0[a]	9.0±5.0	71±7.0
4	75±7.0[b]	14±6.0	12±6.0
10	95±1.0[b]	3.0±1.0	2.0±0.0
50	76±10[b]	1.0±0.0	22±11
100	93±3.0[b]	1.0±0.0	5.0±4.0
200	97±0.0[b]	2.0±0.0	1.0±0.0

[a, b] Similar superscripts indicate no significant difference ($p > 0.05$).

3.5. Discussion

Clavier (1989) proposed that fertilisation in abalone was not effective at all sperm concentrations, with too low or high concentrations resulting in poor fertilisation rates. In this study, sperm concentrations lower than 500 sperm mL^{-1} resulted in hatch-out rates of 50±4%, while concentrations higher that 1 000 000 sperm mL^{-1}, resulted in an increase in larval abnormalities (31±7%). Reduced fertilisation success at low sperm concentrations are thought to be a function of the decreased probability of successful sperm-egg encounters. High sperm concentrations resulting in low hatch-out rates or increased larval abnormalities are considered the result of high sperm lysin concentration, which rapidly dissolves the egg membrane causing polyspermia (Clavier 1989; Encena *et al.* 1998; Babcock & Keesing 1999; Baker & Tyler 2001).

Optimal sperm concentrations have been found to differ between abalone species (**Table 3.5**). In *Haliotis midae,* a sperm concentration of 50 000 sperm mL^{-1} resulted in optimal fertilisation although fertilisation rates were high in the range of 1 000 to 100 000 sperm mL^{-1}. When comparing optimal sperm concentrations between abalone species, it would seem that smaller species require less sperm for optimal fertilisation than larger species. Similarly, larger species produce larger eggs and are commonly found in temperate regions (Clavier 1989; Encena *et al.* 1998; Huchette *et al.* 2004; Al-Rashdi & Iwao 2008). In *H. midae* however, the opposite was found. Shell length and egg size in *H. midae* are similar to that of other temperate species but exhibits a lower optimal sperm concentration.

Table 3.5 Comparison of average sperm concentrations (SC) for optimal fertilisation in selected *Haliotis* species as a function of average animal size (SL = shell length) and correlating egg sizes (ED = egg diameter), as well as origin of species.

Species	Location	SL (cm)	SC (sperm mL^{-1})	ED (µm)	Reference
H. asinina	Philippines	8	50 000	150	Encena *et al.* 1998
H. corrugata	N. America	20	500 000	200	Leighton 1974
H. fulgens	N. America	18	500 000	210	Leighton 1974
H. laevigata	Australia	18	1 000 000	230	Leighton 1974
H. mariae	E. Africa	9	100 000	200	Al-Rashdi & Iwao 2008
H. midae	S. Africa	20	50 000	220	This study
H. rubra	Australia	15	1 000 000	250	Huchette *et al.* 2004
H. rufescens	N. America	25	1 000 000	250	Leighton 1974
H. sorenseni	N. America	20	1 000 000	200	Leighton 1974
H. tuberculata	France	10	500 000	200	Clavier 1989

Differences observed in optimal sperm concentrations between abalone species might thus be a result of variation in sperm morphology and/or sperm-egg binding interaction. Both these factors could be a result of the overlapping distribution ranges and evolutionary divergence of Haliotid species.

It has been suggested that sperm morphology could influence the optimal sperm concentration required for fertilisation in abalone (Grubert *et al.* 2005). Differences in sperm component lengths might give an indication as to why optimal sperm concentrations vary between abalone species (**Table 3.6**).

Table 3.6 Comparison of sperm component lengths of selected *Haliotis* species.

	Sperm component length (µm)				
Species	Acrosome	Nucleus	Midpiece	Total head	Reference
H. asinina	1.0	1.5	0.5	3.0	Suphamungmee *et al.* 2008
H. laevigata	1.4	2.2	0.8	4.4	Grubert *et al.* 2005
H. midae	3.1	2.8	0.1	6.0	Hodgson & Foster 1992
H. rubra	1.3	2.1	0.6	4.0	Grubert *et al.* 2005
H. rufescens	2.5	4.2	0.8	7.5	Lewis *et al.* 1980

A large acrosome could theoretically contain more sperm lysin, implying a lower optimal sperm concentration needed for the disruption of the vitelline envelope (VE). It could thus be plausible that *H. midae* releases more sperm lysin during sperm-egg binding due to its large acrosome. This, however, is not the case when considering the acrosome size of *H. rubra*, which is smaller than that of *H. rufescens* although both these species exhibits the same optimal sperm concentration.

It is further proposed that the shape of the sperm head might influence the penetration ability of the sperm through the VE (Grubert *et al.* 2005). The sperm head of *H. rubra* is blunter than that of *H. rufescens*; suggesting that the latter might be more efficient in penetrating the VE (**Figure 3.2**). Similarly, *H. midae* sperm exhibits a more V-shaped sperm head compared to *H. rufescens*, giving a possible explanation for the variation in optimal sperm concentration between these two species.

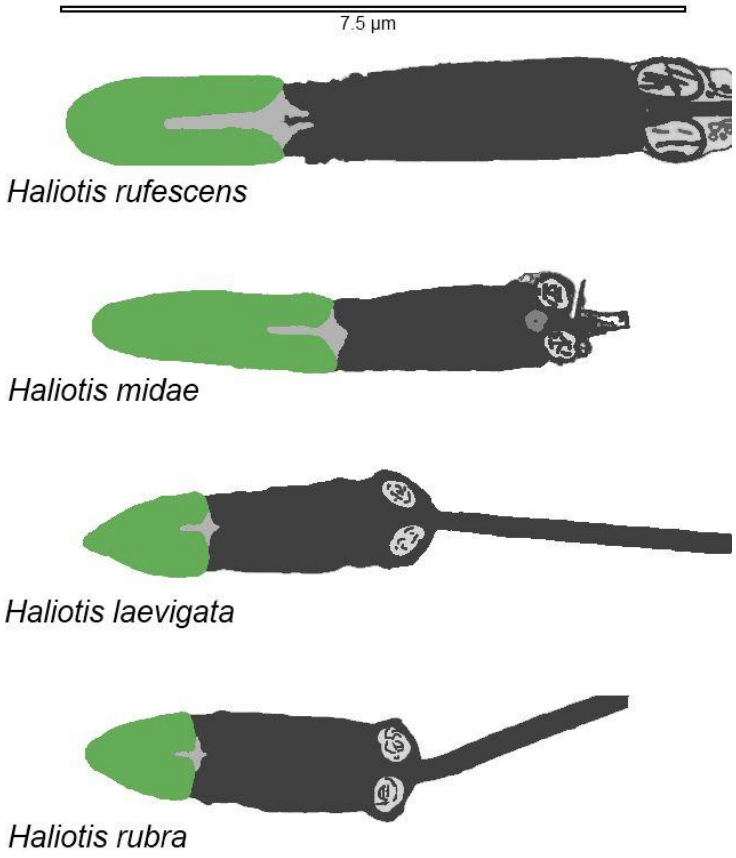

Figure 3.2 Comparison of sperm acrosome lengths of selected *Haliotis* species.

The species-specificity of sperm lysin could also influence the optimal sperm concentration for fertilisation. Sperm lysin potency between abalone species might differ. When considering this, it could be supposed that *H. midae* lysin is extremely effective at low sperm concentrations, reducing the need for high sperm concentrations for successful fertilisation (Grubert *et al.* 2005; Panhuis *et al.*

2006; Aagard *et al.* 2009). This is also reflected in hybridisation experiments between abalone species. It would seem that in some species, sperm lysin more readily dissolves interspecific VE than in reciprocal crosses. Lysin from *H. fulgens* and *H. rufescens* sperm exhibits a 63% sequence identity. Hybridisation experiments with these two species, however, indicated that *H. rufescens* abalone lysin could not dissolve the VE of *H. fulgens*, although *H. fulgens* lysin could partially dissolve the VE of *H. rufescens* when high sperm concentrations were used (Lewis *et al.* 1989; Kresge *et al.* 2000).

Sperm-egg interaction is based on the molecular recognition between gamete surface components i.e. sperm lysin contained in the acrosome and the egg vitelline receptors for lysin (VERLs) distributed across the elevated vitelline envelope (Lewis *et al.* 1989; Swanson & Vacquier 1997). Abalone sperm lysin is 16 kDA acrosomal protein used by sperm to create a hole in the VE, while VERL is a large fibrous glycoprotein composing 30% of the VE and contains approximately 26 tandem repeats of a 153 amino acid lysin-binding domain (Swanson & Vacquier 1997). The number of egg vitelline receptors for lysin (VERLs) on the egg surface might explain the variation in optimal sperm concentrations observed in Haliotids. Two molecules of lysin bind each VERL repeat, resulting in an average of 50 lysins bound per VERL molecule (Swanson & Vacquier 1997). Hence, if an egg contains numerous VERLs, more sperm will be required for optimal intraspecific fertilisations. Although no research has been conducted on egg-sperm binding sites in *H. midae,* it might be possible that *H. midae* eggs possess fewer VERLs than other temperate species, resulting in the low optimal sperm concentration needed for effective fertilisation.

Currently, little is known about the VERLs of abalone species except that it seems to be as species-specific as sperm lysin (Supernault *et al.* 2010). During phylogenetic studies, it was found that the repeat sequences in the VERL become more similar to each other within a species than between any two species. This indicates that the egg components change first during evolution, forcing the sperm to adapt to enable it to bind to the new sequences; thus resulting in a differentiation of the sperm-egg binding system of a species (Vacquier 1998). When this happens in a population, it could lead to two reproductively isolated populations resulting in a subsequent speciation event. In such a case, there will no longer be gene exchange, and each population will embark on an independent evolutionary trajectory resulting in exclusive fertilisation systems (Kresge *et al.* 2000). Such a scenario may explain why *H. midae* and *H. rubra/H. laevigata* possess divergent optimal sperm concentrations for fertilisation even though the Australian endemics share a relatively recent monophyletic origin with *H. midae* (Degnan *et al.* 2006). It could be postulated that reproductive isolation in the “Australian-South African” ancestral species facilitated the sympatric species divergence as a block to hybridisation between the species. On the contrary, *H. midae* and *H. asinina* seem to have similar sperm concentrations for fertilisation; this could be ascribed to convergent evolution: these two species are phylogenetically less related and do not have the same geographic distribution, hence historic hybridisation between these species are highly unlikely; allowing each to separately evolve similar reproductive strategies (Aagard *et al.* 2009).

Optimal egg concentrations for experimental fertilisation have been studied in only few other molluscs including mangrove cupped oyster (*Crassostrea rhizophorae*), giant clam (*Tridacna gigas*) and doughboy scallop (*Chlamys asperrima*) (Crawford *et al.* 1986; Rampersad *et al.* 1994; O'Connor & Heasman 1995). In *Crassostrea rhizophorae* larval survival and egg concentrations were inversely proportional when a range of egg concentrations was used in fertilisation trials (Rampersad *et al.* 1994). In the current study however, the effective egg concentrations ranged from of 10 to 125 mL^{-1}, with optimal hatch-out obtained at a concentration of 50 eggs mL^{-1}. Hatch-out success decreased from 77±6% ($p < 0.05$) at concentrations above 250 eggs mL^{-1}, to 15±2% hatch-out at 500 eggs mL^{-1}. In Pacific oyster (*Crassostera gigas*), it has been suggested that a water-soluble substance, originating from the zygote, rather than oxygen depletion or physical crowding of the eggs (Song *et al.* 2009), causes this deleterious effect of a high egg density. To increase fertilisation rate without compromising hatch-out success, it is advisable to use egg concentrations lower than 125 egg mL^{-1} in *H. midae*.

The effect of egg age is another factor that can influence fertilisation success, as abalone eggs can only be fertilised within a certain period post-spawning. The duration of egg viability is highly variable between abalones, with eggs of some species loosing viability after as little as five minutes post-spawning. In general, however, abalone eggs are capable of being fertilised for up to two hours post-spawning (Leighton 1974; Babcock & Keesing 1999; Ahmed *et al.* 2008).

In hatchery procedures, the effect of egg age might play an important role in determining the optimal time for fertilisation. The effect of ageing on eggs comes into play prior to sperm-egg binding but little is known of the factors that affect egg viability (Baker & Tyler 2001; Zhang 2008). The effect of ageing became evident in this study as egg viability decreased significantly two hours after spawning, with hatch-out rates significantly declining from 65±8% to 48±10% ($p < 0.05$) three hours after spawning. Research on abalone egg age is limited but it is understood that there is a finite period following spawning in which the eggs are capable of being fertilised (Williams & Bentley 2002). Results from this study indicate that *H. midae* eggs should be fertilised as soon as possible after spawning or within the first two hours post-spawning. Similar results to the present study were found for *H. asinina*, which also showed a decrease in egg fertilisability two hours post-fertilisation (Encena *et al.* 1998).

It is crucial to determine the optimal densities of eggs and sperm in a given volume, as many studies do not consider volume when determining optimal gamete ratios for fertilisation (Song *et al.* 2009). In larger volumes, sperm morphology and motility might influence the outcome of fertilisation success. Sperm might lose motility in larger volumes, which would result in the requirement of a higher sperm concentration for successful fertilisation in larger volumes. Fertilisation volumes used for experimental and hatchery procedures will thus influence fertilisation success, although to a lesser degree than egg and sperm ratios.

Fertilisation volumes used in this study resulted in no significant variation between the hatch-out rates, except in volumes of 1 mL, where the number of unfertilised eggs remained high (71±7%). The low number of fertilised eggs can possibly be ascribed to the small volume of sperm used in this volume, i.e. an optimal sperm density of 50 000 sperm mL^{-1} could not be achieved in this volume. The lack of an effect of fertilisation volume on hatch-out success reported in this study correlates with the findings of studies in Pacific oyster, where fertilisation volumes did not significantly affect hatch-out success (Song *et al.* 2009). In *H. midae*, the optimal fertilisation volume for small-scale fertilisations was found to be 200 mL FSW.

3.6. Conclusion

This study reports the development of a species-specific fertilisation protocol for *Haliotis midae*. Fertilisation procedures for *H. midae* have been adopted from other *Haliotis* species, without considering potential differences in biology between species. Results from this study suggest that fertilisation in *H. midae* should be performed within two hours post-spawning to ensure optimal hatch-out rates. It is recommended using sperm at a concentration of 50 000 sperm mL^{-1} while egg concentrations could range between 10 to 125 eggs mL^{-1}. In the current study, optimal fertilisation volumes were found to be 10 mL, 100 mL and 200 mL FSW. Future research needs to investigate the implementation of this fertilisation protocol on the larger scale of hatcheries to determine if the results found in this study are replicable in, and applicable to, commercial hatchery production systems. Factors such as sperm-egg binding sites should also be investigated to evaluate the correlation with optimal sperm concentrations.

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Chapter 4 Determination of larval competence in *Haliotis midae* by using a toxicity assay

4.1. Abstract

This study presents a toxicity assay to evaluate larval competence in *Haliotis midae* by employing dimethyl sulfoxide (Me_2SO) as a chemical toxicant. The median effective concentration (EC_{50}) of Me_2SO for fertilised *H. midae* eggs was 0.7%. This concentration can be used in larval competence assays to determine the ability of larvae to resist any morphological changes induced by this toxicant. Competent larvae, i.e. larvae that will offer a greater degree of resistance to the effects of Me_2SO, will exhibit fewer morphological abnormalities when incubated for 24 hours in 0.7% Me_2SO. This test will enable hatchery managers to assess the relative competence of the larvae, as well as the potential investment of the broodstock female in the eggs. Hatch-out rates below 50% obtained at 0.7% Me_2SO can be related to poor egg quality, indicating larvae with a compromised physiological tolerance to the effects of Me_2SO at its median effective concentration. The toxicity assay can potentially be used to identify larvae, which might not be tolerant to the stress of settlement induction. Evaluating larval competence of abalone will assist hatchery managers in monitoring the reproductive output of individual broodstock females, and in identifying broodstock that consistently produce substandard larvae and can thus be removed from the production system.

4.2. Introduction

In aquaculture, aspects pertaining to reproduction (i.e. broodstock quality, gamete quality, and larval fitness) are important traits to evaluate when implementing and optimising hatchery procedures and output. Assessment of larval competence has become increasingly important in aquaculture operations where hatch-out rates remain variable, even when standardised hatchery management protocols are implemented (Churchill 2003; Sangsawangchote *et al.* 2010). Normally 40% of fertilised eggs can be expected to yield competent larvae in *Haliotis rubra*. Of this, only 10% to 30% have been predicted to settle and reach a minimum weaning size of 1 mm if standard hatchery techniques are used (Heasman & Savva 2007). Although hatch-out rates in *H. midae* remains high (i.e. 80%), subsequent settlement reduces spat yield to approximately 50% with only about 30% of juveniles surviving hatchery cultivation (personal communication Adri Claasen).

Larval competence relates to the physiological ability of larvae to grow and survive under various naturally occurring or induced environmental conditions during their life cycle. Larval competence can be influenced by both endogenous and exogenous factors (Burke *et al.* 2008). Endogenous factors include genotype, parental age and size, egg properties such as the biochemical content of the egg cytoplasm as well as egg size, while exogenous factors are represented by management strategies,

feeding behaviour, and environmental influences (Brooks *et al.* 1997; Kjørsvik *et al.* 2003; Sangsawangchote *et al.* 2010).

Protocols making use of chemical, starvation and osmotic stress resistance methods, have been used to quantify larval competence in various fish and shellfish species, including *Scophthalmus maximus* (turbot), *Perca fluviatilis* (perch), *Ranina ranina* (red frog crab), *Macrobrachium rosenbergii* (giant freshwater prawn), *Scylla serrata* (mud crab), and *Babylonia areolata* (spotted babylon) (Cavalli *et al.* 2000; Hamada *et al.* 2002; Churchill 2003; Kjørsvik *et al.* 2003; Henrotte *et al.* 2010; Sangsawangchote *et al.* 2010).

In perch, larval vigour against osmotic stress provided an indicator of the ability of these larvae to withstand stress, and showed a positive correlation to egg quality (Craik 1985). In turbot, salinity resistance was evaluated and used as a predictive tool to quantify egg quality, as results confirmed that poor egg quality resulted in poor larval viability, with the larvae being less resistant to osmotic stress (Kjørsvik *et al.* 2003).

Tolerance of abalone larvae to stressors have been used for the evaluation of water quality, testing of sterilisation protocols and determining optimal stocking densities. However, no account of stress tests to evaluate larval competence has been reported for any abalone species (Lyon 1995; Conroy *et al.* 1996; Shackleton *et al.* 2002; Huchette *et al.* 2003; Etheridge *et al.* 2004; Tsai *et al.* 2004; Gorski & Nugegoda 2006). Larval tolerance might provide an indication of egg quality, as abalones are lecithotrophic, and larvae are dependent on the biochemical resources that the female invests in the egg cytoplasm. Abalone eggs with a high protein, carbohydrate, and lipid content will supply larvae with sufficient energy sources during development, which in turn will render the larvae more robust against external stressors (Sangsawangchote *et al.* 2010).

Carotenoids, which are dissolved in the lipid droplets of eggs, have also been linked to fertility and embryogenesis, providing antioxidant protection against free radicals during the developmental stages that requires high oxygen metabolism (Craik 1985). In addition to being used as a substrate for metabolism, the lipid reserves, especially the phospholipid component of the egg, are also used to form the structural constituents of the embryo and larval membranes (Henrotte *et al.* 2010). Chemicals that might disrupt these phospholipid membranes can be used as stressors to determine the quality of larval membrane biogenesis. One such chemical is dimethyl sulfoxide (Me_2SO), a chemical substance commonly used as cryoprotectant in cryopreservation since its discovery 50 years ago (Alapati 2009). Dimethyl sulfoxide is an amphipathic solvent with a molecular weight of 78.13 M (Adam *et al.* 1995). It is clinically used as a vehicle for drug delivery in humans due to its dissolving properties, and is nontoxic at low concentrations when applied topically in humans.

To evaluate the effect of various cryoprotectants for cryopreservation on molluscs, cytotoxicity tests indicated that larvae of various species, including *Penaeus indicus* (white prawn), *Evechinus chloroticus* (sea urchin), *Asterina miniata* (starfish) and *Crassostrea gigas* (Pacific oyster) were more resistant to the toxic effect of Me_2SO than fertilised eggs and embryos; possibly due to the decreased permeability of the larval membrane (Newton 1996; Smith *et al.* 2001; Hamaratoğlu *et al.* 2005; Adams *et al.* 2006).

Fertilised eggs of *Haliotis midae* subjected to Me_2SO in similar tests as the above-mentioned studies showed an increase in morphological abnormalities in direct correlation to increasing Me_2SO concentrations (Roux *et al.* 2008). Although Me_2SO proved to be a possible candidate as cryoprotectant for the cryopreservation of *H. midae* fertilised eggs, the significant deformities caused by this toxicant makes it a suitable compound for evaluating the robustness of larvae against toxicants and therefore indirectly, egg quality.

In abalone hatcheries, a simple and effective test is needed to evaluate larval vigour. The aim of this study was to develop a simple toxicity test to quantify larval competence in *H. midae*. Such a test may assist hatchery managers to retrospectively assess egg quality and thus monitor the reproductive output of individual broodstock females. This could support the identification of broodstock, which consistently produce substandard larvae and can potentially be removed from the production system.

4.3. Materials and Methods

Experimental procedures followed in this study are described in Section 1.7. As the standard spawning protocol on farms participating in this study is to spawn animals every six weeks, different animals were used every week during this trial.

4.3.1. Determining the EC50 of Me_2SO

After egg counts, 2 000 pooled eggs were fertilised in tissue culture plates each containing 10 mL FSW using a concentration of 50 000 sperm mL^{-1}. Forty minutes post-fertilisation, with 50% eggs exhibiting a second polar body, FSW was aspirated from the wells. A dilution series of Me_2SO (0.2%, 0.4%, 0.6%, 0.8%, and 1.0%) was freshly prepared with FSW (v/v) and added to the eggs directly after aspiration. Fertilisations were done in triplicate and eggs kept at 18°C in a refrigerated incubator (LM 570-R, MRC).

Twenty four hours post-fertilisation, hatch-out rates were determined microscopically, and recorded as the proportion between the number of hatched larvae, number of abnormal larvae, and number of unfertilised eggs in relation to the total number of eggs used for fertilisation. Larvae were considered

abnormal when they showed irregular shape, abnormal cilia, decreased size, arrested development (one cell stage to immature veliger), or if larvae were trapped in the egg membrane. The different types of abnormalities were grouped into a single category.

4.3.2. Statistical analysis

All data on hatching success was pooled and expressed as percentages. To ensure a normal distribution of the data, all proportional data were arcsine transformed using Statistica 7 before a paired t-test was applied. Analysis of variance was used to determine significant differences between treatments. Results are presented as the mean percentages with standard errors. This experiment was repeated over three consecutive spawnings to obtain up to nine data points per toxicant treatment.

4.4. Results

Hatch-out rates did not differ ($p > 0.05$) between the control (0.0% Me_2SO; 90±1% hatch-out) and 0.2% Me_2SO (89±2%) (**Table 4.1**).

Table 4.1 Hatch-out rates of normal and abnormal *H. midae* larvae (mean±SEM) obtained from embryos exposed to different concentrations of Me_2SO.

Me_2SO (%)	**Mean normal larval hatch-out rate (%)**	**Mean abnormal larval hatch-out rate (%)**
0.0	90±1.0[a]	5.0±1.0
0.2	89±2.0[a]	8.0±1.0
0.4	81±3.0[a]	15±2.0
0.6	65±3.0[b]	33±3.0
0.8	25±3.0[c]	72±4.0
1.0	10±2.0[d]	87±3.0

[a, b, c, d] Similar superscripts indicate no significant difference ($p > 0.05$).

A significant decrease ($p < 0.05$) in normal larvae was observed with an increase in toxicant concentrations of 0.4% Me_2SO to 1.0% Me_2SO, with the number of abnormal larvae progressively increasing in a linear fashion as the toxicant concentration was increased from 0.4% Me_2SO to 1.0% Me_2SO (**Figure 4.1**). This inversely correlated toxic effect of Me_2SO reached a median point between 0.6% Me_2SO (65±3% normal hatch-out) and 0.8% Me_2SO (25±3% normal hatch-out), indicating the effective median concentration after a 24 hour incubation period in Me_2SO (EC_{50} – 24h) for fertilised *H. midae* eggs was 0.7%.

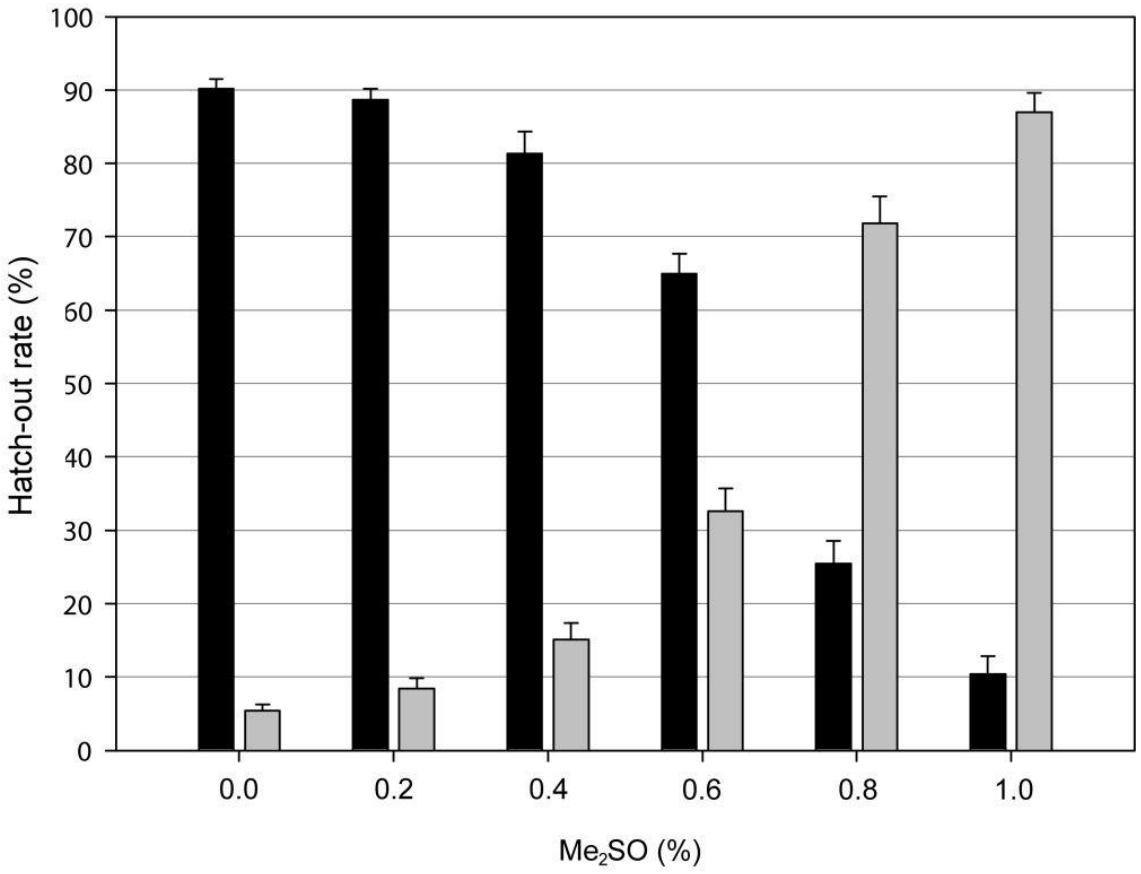

Figure 4.1 The influence of 24 hour embryonic exposure to different concentrations of Me_2SO (dimethyl sulfoxide) on subsequent hatch-out rates of *H. midae* larvae. (Normal larvae = black bars; Abnormal larvae = grey bars).

4.5. Discussion

The early developmental stages of marine fish and shellfish have been shown to be highly sensitive to external factors such as water quality. This indicates a basis for using fertilised eggs of these species in stress or toxicity tests with successful embryogenesis as the end point to monitor environmental pollutants and water quality (McGibbon & Moldan 1986; Conroy *et al.* 1996; Etheridge *et al.* 2004; Jorge & Moreira 2005). The toxic effects of various chemicals observed in these environmental studies, lead to the development of toxicity tests to determine and quantify larval robustness against external stressors (Cherr *et al.* 1990; Cavalli *et al.* 2000; Churchill 2003; Henrotte *et al.* 2010).

Toxicity tests in abalone have been limited to the evaluation of water quality, testing of sterilisation protocols as well as determining optimal stocking densities (Lyon 1995; Liao & Lin 2001; Huchette *et al.* 2003; Gorski & Nugegoda 2006). To my knowledge, no research has been done to evaluate the

robustness of larvae by employing toxicity tests. In this study, a simple acute static stress test was developed and used to obtain data on the larval competence of *H. midae*.

The EC_{50} – 24h of Me_2SO was determined at 0.7% for fertilised *H. midae* eggs. At this concentration, 50% of *H. midae* larvae exhibited abnormalities. These abnormalities can be ascribed to the amphiphilic properties of Me_2SO, which strongly interacts with the phospholipid membrane of the fertilised egg. This interaction changes the partition of hydrophobic molecules between the cell membrane and its external environment, causing dehydration of the phospholipid membrane and in some cases disruption of the membrane (Nascimento *et al.* 2005; Qi *et al.* 2008). Dimethyl sulfoxide is also known to inhibit catalase; an important enzyme for lipid synthesis. The inhibition of this enzyme can cause morphological damage to the membrane structure of the developing larvae (Adam *et al.* 1995; Adams *et al.* 2006).

The biochemical components of the egg cytoplasm can provide larvae with mechanisms to counteract chemical stressors. In *Danio rerio* (zebra fish) embryos, Me_2SO inhibited lipid synthesis, but the effect was reduced in larvae as the lipid membrane became less permeable to the toxicant (Adam *et al.* 1995). A similar pattern was observed in *Crassostrea rhizophorae* (mangrove cupped oyster), where larvae were more resistant to the toxicity of Me_2SO than embryos (Nascimento *et al.* 2005). Sangsawangchote *et al.* (2010) reported that the endogenous egg reserves of *Babylonia areolata* (spotted Babylon) might have improved the survival rate of larvae during a starvation stress test. In their study, they concluded that lipids were utilised during embryogenesis for membrane development, and that increased lipid content in the egg cytoplasm would improve the survival rate of developing spotted Babylon larvae by supplying an energy source, but also by contributing to the structure and efficacy of the embryonic membrane to counteract external stress.

In the present study, *H. midae* larvae with less morphological abnormalities (i.e. normal hatch-out rates of above 50%) at a concentration of 0.7% Me_2SO potentially had greater tolerance to the toxic effects of Me_2SO. This heightened tolerance might be the result of enhanced egg biochemical content e.g. higher lipid levels in these eggs whereas increased abnormalities might be correlated to compromised biochemical contents, rendering the developing larvae less competent to tolerate the morphological breakdown caused by the Me_2SO. In the presence of a chemical stressor, developing larvae will require more energy to resist stress and in turn, the higher metabolic rate could produce excess oxygen radicals. Increased carotenoid content in the eggs could thus afford larvae increased protection against free radical damage, and will minimise morphological abnormalities in the larval stages (Sawanboonchun *et al.* 2008).

When *H. midae* embryos were subjected to the chemical toxicity of Me_2SO, the increased larval abnormalities might have been a consequence of a low carotenoid content in the eggs, as Me_2SO causes the disruption of the lipid membranes resulting in the leaching out of carotenoids. The lower

carotenoid levels could potentially render the developing larvae less resistant to the damage caused by free radicals, while larvae from eggs with higher carotenoid levels could potentially have an improved ability to withstand the damage caused by free radicals, and therefore offered more resistance to the chemical stressor.

When the toxic stress effect of Me_2SO was evaluated on sea urchin larvae, it was found that morphological damage caused by Me_2SO to the lipid membranes, resulted in the leaching of carotenoids from the embryos, affecting the robustness of the larvae to respond to stress. The study also found that larvae produced by certain females seemed to have an increased ability to recover from the toxic effect of Me_2SO, indicating the contribution of the egg components to larval competence (Adams 2003).

4.6. Conclusion

A toxicity test employing Me_2SO as an external stressor was developed to determine larval competence in *H. midae*. At concentrations of 0.7% Me_2SO, 50% of larvae exhibited morphological abnormalities, indicating a critical point at which larvae cannot resist the toxic effect of Me_2SO. Larvae with higher robustness against the toxic effects of Me_2SO would exceed this critical point by showing less morphological abnormalities when incubated for 24 hours in Me_2SO. A higher lipid and carotenoid content potentially contributed to this enhanced ability of larvae to withstand the toxic effects of Me_2SO. Evaluating larval competence by means of the Me_2SO toxicity test could provide hatchery managers with a tool to quantify larval fitness, and indirectly evaluate the maternal investment in egg quality, thus identifying female broodstock that produce eggs of better quality.

The toxicity test developed in the current study could be incorporated into current hatchery procedures since it can be run concurrently after fertilisation of an egg batch. Larval competence could then be determined 24 hours post-fertilisation, allowing the hatchery manager sufficient time to evaluate larval fitness before continuing with settlement. In eliminating larvae with low competence, settlement rates can be increased which will consequently contribute to optimising and improving hatchery output.

In future studies, the lipid and carotenoid levels in eggs as well as in the resulting larvae, need to be determined and evaluated to verify the relationship between larval competence and the biochemical content of the eggs. It would also be of value to monitor the cellular development of the larvae when subjected to toxicants to determine the exact mechanisms with which the toxicant disrupts larval structures.

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Chapter 5 Hybridisation of *Haliotis midae* and *H. spadicea* using biopsied spermatozoa

5.1. Abstract

This study reports on the production of viable *Haliotis midae* X *Haliotis spadicea* hybrid larvae using biopsied *H. spadicea* spermatozoa to fertilise spawned *H. midae* eggs. To our knowledge, this is the first account of hybridisation between these species, as well as the first description of a non-invasive gonad biopsy in abalone.

Firstly, to determine the use of a gonad biopsy for successful fertilisation, spawned *H. midae* eggs were fertilised using biopsied *H. midae* spermatozoa. This resulted in an average hatch-out rate of 66.9±8.5% although hatch-out success between individual animals was highly variable (96.3±1.9% to 31.5±1.1%) due to unknown spermatozoa concentrations in biopsy extracts. When using spawned *H. midae* sperm, hatch-out rates of 93.5±2.0% were obtained. Males subjected to repetitive biopsies showed no signs of injury or mortality.

Secondly, the hybridisation potential between *H. midae* X *H. spadicea* was determined by obtaining biopsied spermatozoa from *H. spadicea*. Interspecific fertilisations using spawned *H. midae* eggs resulted in the production of viable hybrid larvae. Interspecific hatch-out rates (3.9±0.9%) were significantly lower ($p < 0.05$) than that of intraspecific fertilisations (66.9±8.5%), indicating a degree of gamete incompatibility between these two species.

The genetic status of the *H. midae* X *H. spadicea* hybrid was verified by quantitative real-time polymerase chain reaction (QPCR) and high-resolution melt-curve (HRMC) analysis. PCR products were sequenced to verify HRMC results and confirmed the conclusion of a single base pair (bp) difference in the lysin gene region between *H. midae* and *H. spadicea*; as well as the presence of both genotypes in hybrid larvae.

5.2. Introduction

Abalone hybrids are found naturally (Owen 1971; Arai *et al.* 1986; Brown 1995) and have also been produced by experimental cross-breeding (Leighton & Lewis 1982; Hoshikawa *et al.* 1998; Ibarra *et al.* 2005; Ahmed *et al.* 2008). Hybrids have also been produced in various other mollusc species i.e. oyster (*Crassostrea gigas* X *C. rivularis*), mussel (*Mytilus edulis* X *M. trossulus*), prawn (*Penaeus monodon* X *P. esculentus*) and sea urchin (*Strongylocentrotus nudus* X *S. intermedius* X *Anthocidaris crassispina*), especially due to the potential cumulative effect of heterosis (hybrid vigor) (Allen & Gaffney 1993; Benzie *et al.* 1995; Launey & Hedgecock 2001; Toro *et al.* 2004; Ding *et al.* 2007).

In nature, pre- and post-zygotic barriers could inhibit hybridisation between species. Pre-zygotic mechanisms include ecological isolation (geographic separation and distance between interspecific species), temporal isolation (different mating or spawning seasons), behavioural isolation (incompatible courtship), mechanical isolation (incompatible genitalia), and gametic isolation (incompatible gametes). Most of these pre-zygotic barriers can however be overcome in aquaculture through artificial fertilisation techniques.

One of these techniques includes gonad biopsies for obtaining gametes, especially in species including common shrimp (*Penaeus setiferus*), whitelegged shrimp (*Penaeus vannamei*), kuruma prawn (*Penaeus japonicus*), giant clam (*Tridacna gigas*), asian moon scallop (*Amusium pleuronectes*), mangrove cupped oyster (*Crassostrea rhizophorae*), black-lipped pearl oyster (*Pinctada margaritifera*), freshwater mussel (*Hyridella depressa*), sea cucumber (*Cucumaria frondosa*) and windowpane shell (*Placuna placenta*) where spawning is difficult to induce (Crawford *et al.* 1986; Belda & Del Norte 1988; Rampersad *et al.* 1994; Hamel & Mercier 1996; Suitoh *et al.* 1996; Madrones-Ladja 1997; Byrne 1998; Acosta-Salmon & Southgate 2004). Biopsy sampling involves the use of conventional syringes and needles or specialised human biopsy needles to extract small tissue samples from target organs without harming the organism (Anderson *et al.* 1984; Crawford *et al.* 1986; Acosta-Salmon & Southgate 2004). Gonad biopsies have resulted in successful fertilisation in various mollusc species and are a prospective method to obtain gametes for interspecific *in vitro* fertilisation (Misamore & Browdy 1997).

In South Africa, considerable research has been done on *Haliotis midae* to improve commercial production by addressing problems such as slow and variable growth (Newman 1966; Raemaekers & Britz 2009; Simon *et al.* 2009; Slabbert *et al.* 2009; Vosloo *et al.* 2009). As *H. spadicea* is not cultured, aspects of its growth rate, meat quality, and yield is unknown. Studies found that both species reach sexual maturity at the age of three years, with *H. spadicea* spawning once a year during December, and *H. midae* spawning during April and September. This asynchronous spawning pattern limits the possibility of natural hybridisation, even though these species cohabit in the intertidal zone (Muller 1984; Toro *et al.* 2004).

A preliminary evaluation of the crossbreeding potential between *H. spadicea* and *H. midae* could lay the foundation for future experiments in determining the effect of heterosis on these species. Such crossbreeding could also result in sterile hybrids, which has been reported to exhibit enhance growth rates as little to no investment is made into gonad development (Ibarra *et al*. 2005; Allsop *et al*. 2010). Since *H. spadicea* is not a cultured species, these animals cannot be chemically induced to spawn. By developing a biopsy method to collect gametes, this problem can be overcome. It is however crucial that collection is non-destructive, as culling animals for gonad retrieval could eliminate possible valuable animals.

In studies on hybridisation it is imperative that hybrids should be genetically verified to rule out the possibility of gamete contamination. Various techniques have been developed for the genetic verification of hybrids including allozyme analysis, microsatellite markers (SSRs), random amplified polymorphic DNA (RAPDs) and restriction fragment length polymorphisms (RFLPs) (Goldstein *et al.* 1995; Sweijd *et al.* 1998; Ahmed *et al.* 2008). A recent technique to distinguish abalone hybrids has been developed based on the species-specific nature of mutations found in the sperm lysin gene by means of high-resolution melt curve analysis (HRMC) (Sweijd *et al*. 1998; Slabbert 2010; Supernault, *et al*. 2010).

The aims of this study were firstly to determine the fertilisation potential of biopsied spermatozoa from *H. midae* and to establish the effect of repetitive biopsies on the survival of *H. midae* males. The second aim was to produce viable hybrid larvae using biopsied spermatozoa from *H. spadicea* and spawned eggs from *H. midae* and verification of hybrid larvae using HRMC analysis.

5.3. Materials and Methods

5.3.1. Obtaining spawned H. midae *gametes for control fertilisations*

Experimental procedures used in obtaining spawned *H. midae* gametes for control fertilisations in this study are described in Section 1.7. After egg counts, 30 eggs were fertilised in tissue culture plates each containing 1 mL FSW using a concentration of 50 000 sperm mL^{-1}. Fertilisations were done in triplicate and eggs kept at 18°C in a refrigerated incubator (LM 570-R, MRC). Twenty four hours post-fertilisation, hatch-out rates were determined microscopically, and recorded as the proportion between the number of hatched larvae, number of abnormal larvae, and number of unfertilised eggs in relation to the total number of eggs used for fertilisation. Larvae were considered abnormal when they showed irregular shape, abnormal cilia, decreased size, arrested development (one cell stage to immature veliger), or if larvae were trapped in the egg membrane. The different types of abnormalities were grouped into a single category.

5.3.2. Obtaining biopsied H. midae *sperm for intraspecific fertilisations*

Testicular tissue was biopsied from mature two year old *H. midae* males (n = 4), which were obtained from the commercial production line of HIK Abalone Farm. These animals had an average weight and shell length of 12±1.5 g and 48±3.0 cm, respectively, and were cultured according to the methods detailed in Section 7.1.

Spermatozoa were aspirated by penetrating the testis with a 20 gauge needle fitted to a syringe filled with 0.1 mL FSW (**Figure 5.1**). By injecting the FSW into the testis, testicular tissue was slowly

extracted and transferred to a tissue culture plate (Sigma). Biopsy extracts were evaluated for the presence of spermatozoa using an inverted microscope (IX51 Inverted Fluorescent Microscope, Olympus).

Due to difficulty in separating spermatozoa from biopsied tissue and germ cells, fertilisation was achieved by adding 50 µL of the biopsy extract to 30 spawned *H. midae* eggs obtained from control animals. Embryo incubation and the enumeration of hatch-out rates (of normal and abnormal larvae) followed the protocols given in Section 5.3.1.

Figure 5.1 The penetration of the testis (in the conical area) of a *Haliotis midae* male to obtain sperm by means of biopsy, using a syringe filled with 0.1 mL filtered seawater (FSW), and fitted with a 20 gauge needle.

After the first biopsy, animals were tagged and returned to the commercial system and maintained under farm conditions. Three weeks later, the animals were retrieved and survival recorded. The experimental animals were monitored for survival two months after the first biopsy, and then returned to the commercial production line.

5.3.3. Biopsy extraction of H. spadicea *sperm for interspecific fertilisations*

Two *H. spadicea* males with an average weight and shell length of 127±15 g and 9.5±1.5 cm respectively, were collected from the intertidal pools of Hermanus Bay. The animals were cultured in oyster net baskets suspended in concrete tanks in isolation from production animals to limit bio-contamination. Seawater at flow rates of 90 L h^{-1} were circulated through the tanks and water

temperature varied between 17°C to 19°C. The animals were fed *Ecklonia maxima* or *Laminaria pallida,* depending on availability.

To evaluate the hybridisation potential of *H. spadicea* and *H. midae*, spermatozoa were aspirated from *H. spadicea* males as described in Section 5.3.2. Biopsied spermatozoa from *H. spadicea* were used for interspecific fertilisations by adding 50 µL of the biopsy extract to 30 spawned *H. midae* eggs obtained from control animals. Embryo incubation and the enumeration of hatch-out rates (of normal and abnormal larvae) followed the protocols given in Section 5.3.1.

After biopsies, the animals were tagged and returned to the isolated tanks and maintained under farm conditions. Three weeks after the first biopsy, the animals were retrieved and survival recorded. The experimental animals were monitored for survival two months after the first biopsy, and then returned to the intertidal pools.

5.3.4. Genetic verification of H. spadicea *X* H. midae *hybrid larvae*

Epipodial tissue was taken from four female *H. midae* females and two male *H. spadicea* used in the interspecific fertilisation trials for the purpose of DNA (deoxyribonucleic acid) sequencing. Twenty four hour old hybrid larvae were also collected and genomic DNA extracted following the protocol of Saghai-Maroof *et al.* (1984).

Primers specific to abalone lysin were used to amplify a 120 bp product (Sweijd *et al.* 1998) from samples consisting of one *H. midae* and one *H. spadicea* specimen, a mixture of DNA from these two individuals (hybrid control) and the larvae sampled during the interspecific cross.

Quantitative real-time PCR and HRMC analysis was performed using a Rotor-Gene 6000 PCR machine and carried out in a final volume of 25 µL under the following conditions: 1x SensiMix HRM (Quantace), 1 µL EvaGreen dye, 5 µM of each primer and 40 ng of DNA template. Cycling conditions were initial denaturation and activation at 95°C for 10 minutes followed by 50 cycles of 95°C for 10 seconds, 53°C for 20 seconds and 72°C for 30 seconds. Analysis of HRMC was done using the Rotor-Gene 6000 Series Software version 1.7 after the temperature was raised from 70°C to 90°C at 0.1°C increments and a hold of 2 seconds for each step. Thereafter, PCR products were sequenced as verification of the HRMC results, using the 3730xl DNA Analyzer (Applied Biosystems). Sequencing was conducted via standard Sanger sequencing chemistry (BigDye® terminator V3.1 cycle sequencing kit, Applied Biosystems) and sent to the Stellenbosch University Central Analytical Facility (DNA sequencing unit) for capillary electrophoresis using the 3730xl DNA Analyzer.

5.3.5. Statistical analysis

To ensure a normal distribution of the data, all proportional data were arcsine transformed using Statistica 7 before a paired t-test was applied. Analysis of variance was used to determine significant differences between treatments. Results are presented as the mean percentages with standard errors. As this experiment was a preliminary evaluation of hybridisation potential, no repeats were performed.

5.4. Results

5.4.1. Spawned vs. biopsied fertilisations in H. midae

When evaluating biopsy extracts microscopically, semi-flagellated and immotile spermatozoa, surrounded by testicular tissue and germ cells were observed (**Figure 5.2**). Despite this immobility, when using biopsied spermatozoa and spawned eggs, a mean hatch-out rate of 66.9±8.5% was obtained. Individual males however, gave highly variable hatch-out rates (31.5% to 96.3%) when biopsied spermatozoa were used.

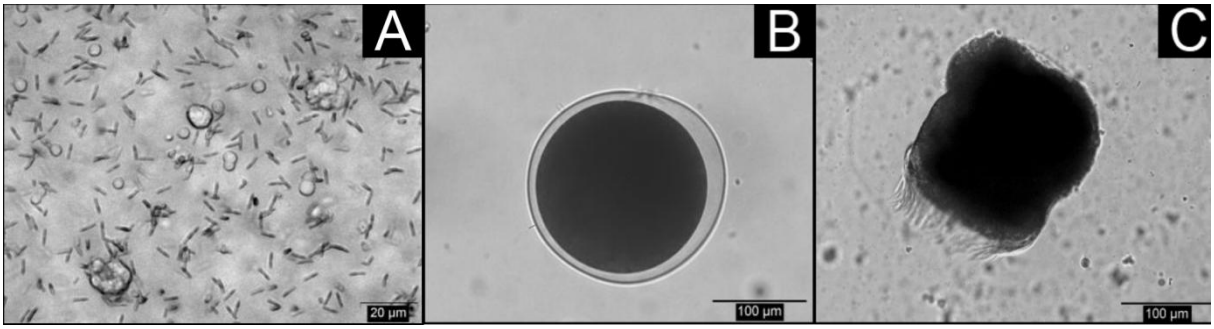

Figure 5.2 *Haliotis midae* biopsy extracts containing spermatozoa (A) used to fertilise spawned *H. midae* eggs (B) and resulting in viable larvae (C).

Fertilisation trials using spawned *H. midae* sperm and eggs resulted in an average hatch-out rate of 93.5±2.0 % (**Table 5.1**). No detrimental effects or mortalities were observed two months after the initial biopsy in any of the males used for repetitive biopsies.

Table 5.1 Hatch-out rates (mean±SEM) obtained from fertilisations using biopsied spermatozoa and spawned eggs from *H. midae* vs. control fertilisations using spawned *H. midae* gametes.

	Animal identification				
	Control	**Hm1**	**Hm2**	**Hm3**	**Hm4**
Origin of spermatozoa	Spawned	Biopsied	Biopsied	Biopsied	Biopsied
Hatch-out rate (%)	93.5±2.0[a]	96.3±1.9[a]	31.5±1.1[b]	93.5±2.0[a]	47.9±4.4[b]

[a, b] Columns with different superscripts differ significantly ($p < 0.05$)

5.4.2. Interspecific fertilisations using biopsied H. spadicea *spermatozoa and spawned* H. midae *eggs*

An average hatch-out rate of 3.9±0.9% was achieved when using biopsied *H. spadicea* spermatozoa to fertilise spawned *H. midae* eggs. This was significantly ($p < 0.05$) lower than the hatch-out success obtained for the intraspecific fertilisation using biopsied *H. midae* spermatozoa and spawned *H. midae* eggs (66.9±8.5%).

5.4.3. Genetic verification of H. spadicea *X* H. midae *hybrid larvae*

Larvae were classified as hybrids based on the results of the HRMC analysis presented in **Figure 5.3**, with *H. midae*, *H. spadicea* and the hybrid control genotypes being clearly discernible. Sequencing results confirmed this conclusion showing a single base-pair (single nucleotide polymorphism: SNP) difference between *H. midae* and *H. spadicea,* as well as the presence of both genotypes in the hybrid larvae samples (**Table 5.2**).

Table 5.2 Sperm lysin nucleotide sequence of *H. midae* and *H. spadicea* and resultant sequence of hybrid *H. midae* X *H. spadicea* larvae where R is the degenerative position of the SNP and can be either G or A.

Species	**Sperm lysin sequence**
H. midae	CTGGATGGAGCCAATGACGTCATCCCAGC**G**CGGGGTGGCCAGATTTTTCAC
H. spadicea	CTGGATGGAGCCAATGACGTCATCCCAGC**A**CGGGGTGGCCAGATTTTTCAC
H. midae X *H. spadicea*	CTGGATGGAGCCAATGACGTCATCCCAGC**R**CGGGGTGGCCAGATTTTTCAC

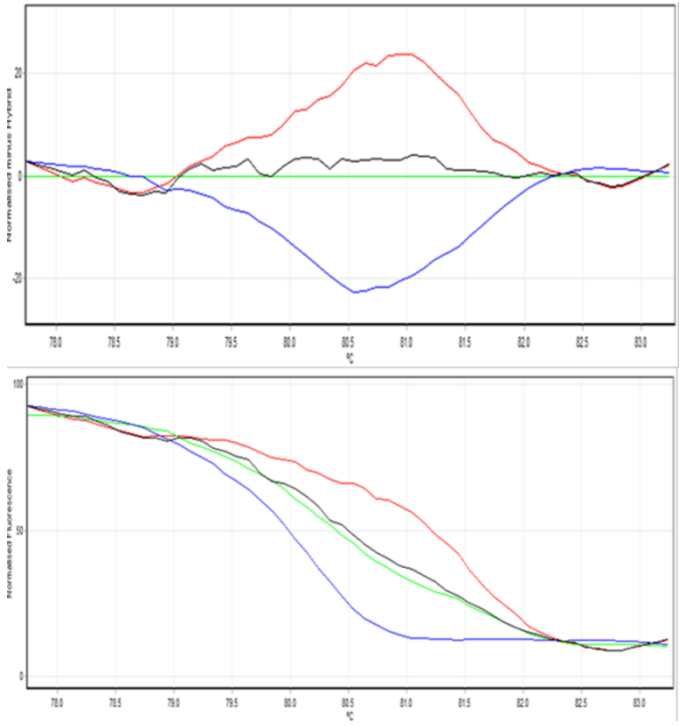

Figure 5.3 Genetic verification of *H. midae* X *H. spadicea* hybrid larvae. The normalised graph (top) and difference graph (bottom) of the high resolution melt curve analysis of sperm lysin single nucleotide polymorphism (SNP) for *H. midae* (red), *H. spadicea* (blue), hybrid control (green) and the *H. midae* X *H. spadicea* hybrid (black).

5.5. Discussion

A biopsy technique was developed in *H. midae* to aspirate spermatozoa for intraspecific fertilisations with spawned *H. midae* eggs. This artificial fertilisation technique eliminated the need for culling animals to obtain gametes. Results indicated that biopsied spermatozoa were able to fertilise spawned eggs, although individual males gave highly variable hatch-out rates (31.5% to 96.3%) compared to that of control fertilisations (93.5±2.0%). This is similar to results obtained in *H. asinina*, where it was shown that spawned and testicular spermatozoa exhibited comparable average fertilisation potential (Suphamungmee *et al.* 2008).

The variation in hatch-out rates observed in this study might be due to the unknown concentration of spermatozoa in biopsy extracts. By using enriched spermatozoa, hatch-out rates could be increased and standardised. Suphamungmee *et al.* (2008) increased spermatozoa concentrations in cell suspension from macerated abalone testes by separating testicular spermatozoa from other germ cells by a discontinuous Percoll gradient centrifugation. Two populations of cells were achieved; one

sedimented at the interphase between 40% and 60% Percoll (composed mainly of germ cells) and the enriched spermatozoa at the bottom.

In the present study, spermatozoa in biopsy extracts from both *H. midae* and *H. spadicea* showed similar morphology and proved to be viable and able to fertilise spawned *H. midae* eggs. As *H. spadicea* is not a cultivated species and induced spawning has not yet been attempted, the biopsy technique developed in this study overcame the pre-zygotic barriers between these two species and interspecific fertilisations were achieved. When using biopsied *H. spadicea* spermatozoa and spawned *H. midae* eggs, low hatch-out rates (3.9±0.9%) were achieved, compared to intraspecific fertilisations with biopsied spermatozoa and spawned eggs from *H. midae* (66.9±8.5%). This significant difference in hatch-out rates ($p < 0.05$) could be ascribed to gamete incompatibility between these two species.

Gamete compatibility lies in the species-specificity of the interaction between the sperm lysin and egg vitelline layer (EVL). Normally, the physical block to fertilisation is overcome by intraspecific sperm lysin, which completely solubilises the EVL. During hybridisation however, interspecies lysin is in some instances incapable of fully solubilising the EVL (Lewis *et al.* 1989). The pronounced decrease in hatch-out rates observed in the interspecific fertilisations between *H. midae* and *H. spadicea*, in comparison to intraspecific fertilisations, is indicative of a high degree of gametic incompatibility between these two species.

This is due to variations in the molecular weight and genetic composition of the sperm lysin between species, and suggests that *H. midae* EVL is less soluble to *H. spadicea* lysin. Reciprocal crosses could potentially lead to higher hatch-out rates as it has been shown that lysin is unequal in specificity in some instances (Leighton & Lewis 1982; Lewis *et al.* 1989; Hoshikawa *et al.* 1998; Ahmed *et al.*, 2008). Until reciprocal crosses are done, *H. spadicea* EVL solubility to *H. midae* lysin cannot be predicted. To test this it would be necessary either to develop a spawning protocol for *H. spadicea*, or to develop a biopsy technique to extract eggs from abalone ovaries.

The interspecificity of *H. spadicea* lysin might also be overcome by increasing the concentration of *H. spadicea* sperm in interspecies fertilisation. Leighton and Lewis (1982) found that sperm concentrations had to be ten times higher for interspecific fertilisations than in intraspecific fertilisations. Luo *et al.* (2009) confirmed this by investigating hybridisation success among *H. discus hannai* and *H. gigantea*. Results from their study indicated an optimal sperm concentration of 4.66×10^7 sperm mL^{-1} for hybridisations between female *H. discus hannai* X male *H. gigantea* and 2.6×10^7 sperm mL^{-1} for the reciprocal crosses (i.e. a 100 fold higher than that required for intraspecific fertilisation).

To confirm successful hybridisation, it is crucial to verify hybrid status by genetic or biochemical evaluation. In hybridisations studies done on various *Crassostrea* species, for example, the genetic

status of presumed hybrids was not evaluated and hybrid status could thus not be confirmed, as gamete contamination could not be excluded (Gaffney & Allen 1993). Various techniques have been developed for the genetic verification of hybrids i.e. allozyme analysis, microsatellite markers, RAPD, and RFLP markers (Goldstein *et al.* 1995; Sweijd *et al.* 1998; Wan *et al.* 2001; Ahmed *et al.* 2008). In this study, a modified protocol was developed using HRMC for *H. midae* X *H. spadicea* hybrid identification. High-resolution melt-curve analysis has been used successfully in previous studies to identify fish species or identify fish mitochondrial haplotypes (Wattanabe *et al.* 2004; Dalmasso *et al.* 2007; Haynes *et al.* 2009). For HRMC, intercalating dyes are used that bind specifically to double-stranded DNA, and when bound, fluoresce. In the absence of double-stranded DNA, these dyes have nothing to bind to and thus only fluoresce at a low level (Wattanabe *et al.* 2004; Dalmasso *et al.* 2007; Haynes *et al.* 2009). The HRMC process starts by the precise warming of the amplicon (i.e. a segment of the targeted gene that has been amplified by PCR). During this process, the melting temperature of the amplicon is reached and the double-stranded DNA separates. This results in a decrease in fluorescence as double-stranded DNA denatures and forms single-stranded DNA, to which no dye is bound. The HRMC camera measures the decrease in fluorescence and plots this data as a graph known as a melt curve, showing the level of fluorescence vs. the temperature. By employing this technique, hybrids produced from interspecific crosses can be confirmed (Reed *et al.* 2007; Slabbert 2010). The high-resolution melt-curve analysis presented in this study rapidly and accurately verified the hybrid status of the larvae resulting from the interspecific cross.

5.6. Conclusion

This study found that successful hybridisation could be achieved by using a biopsy method to aspirate spermatozoa from *H. spadicea* for interspecific fertilisation of spawned *H. midae* eggs without detriment to the donor males. Obtaining spermatozoa via biopsy without mortalities could assist the abalone industry to assess maturity of animals and determine fertilisation potential of selected broodstock. This in turn will accelerate breeding programmes that are currently hampered by long generation times.

Future research in the area of hybridisation between *H. spadicea* and *H. midae* should focus on purifying spermatozoa from testicular germ cells in biopsy extracts to enable the determination of spermatozoa concentrations. This could enhance the hatch-out rates of fertilisations using biopsied spermatozoa. It would also be beneficial to evaluate the potential to extract eggs from female gonads especially for the investigation of reciprocal crosses between *H. spadicea* and *H. midae*.

The hybrid verification method developed in this study could also be applied in other abalone species specifically as well as molluscan species in general to determine the presence of hybrid larvae in breeding experiments.

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Chapter 6 General conclusion

This study presents novel information on the development and optimisation of hatchery techniques for the cultivation of the South African abalone, *Haliotis midae*. These include;

- a description of the onset of sexual maturity,
- development of a fertilisation protocol specifically for *H. midae*,
- the evaluation of larval competence
- and determining the hybridisation potential between *H. midae* and *H. spadicea*

A gonad biopsy technique was also developed and proved effective in the collection of spermatozoa without detrimental effects on the donor animal. This method was employed to extract spermatozoa from an uncultivated abalone species for successful hybridisation experiments. The method can also be used to microscopically determine sexual differentiation in juvenile abalone.

6.1. Gonadogenesis and attainment of sexual maturity in *Haliotis midae*

The assessment of onset of maturity as well as the characterisation of gametogenesis could assist hatcheries in selecting potential broodstock based on gonad structure. Current practise on most abalone farms is to select cultivated animals as broodstock from the age of three to four years (i.e. 45 mm to 85 mm shell width), when the gonads become externally visible. These animals are then conditioned for spawning induction for up to six months. By determining gender and age of sexual maturity at an earlier stage, the conditioning period could be shortened and the reproductive potential of the selected animals evaluated earlier.

Histological evaluation of gonad tissues, based on a new gametogenesis classification system developed here, showed that gonad development begins at an age of 12 to 18 months in cultivated abalone (i.e. a shell width of 20 mm to 25 mm), with 50% sexual maturity attained at 18 months of age (i.e. 25 mm shell width) in males and at 24 months (i.e. 30 mm shell width) in females. This suggests that cultivated animals can be selected for broodstock conditioning from two years of age instead of three to four years, as is currently the case.

However, as gonad colour is not yet externally visible at this age, it may not possible to determine the number of male and females selected for conditioning. This could be overcome by employing the needle biopsy technique developed in this study.

6.1.1. Recommendations and future studies

It is recommended to determine the gender of 24 month old *H. midae* microscopically by sampling tissue from immature gonads using the needle biopsy developed in this study. At this age, the various stages of gametogenesis should be identifiable. When gender is established, these animals can be introduced into the broodstock conditioning system, as most of them will be at 50% sexual maturity.

Future studies on this species should include the evaluation of the effect of early conditioning on gonad development. As results from this study only represent animals obtained from one farm, more studies on the attainment of sexual maturity should include animals from various farms as location and production systems might influence gonad development.

6.2. Species-specific fertilisation protocol for *Haliotis midae*

The development of a species-specific fertilisation protocol for *H. midae* is essential to optimise hatchery output. Hatch-out rates on South African abalone farms are on average 80%. This number decreases to 50% at settlement, with an eventual 30% going into production (personal communication Adri Claasen, November 2010). To increase fertilisation success, it is necessary to determine the optimal gamete concentrations, time for fertilisation as well as fertilisation volume specific for a species.

As optimal sperm concentration varies greatly between abalone species (Clavier 1989; Encena *et al.* 1998; Babcock & Keesing 1999; Baker & Tyler 2001; Grubert *et al.* 2005), it was crucial to determine the optimal sperm concentration for fertilisation specifically for *H. midae*. In the current study, this optimal sperm concentration for fertilisation of *H. midae* eggs was 50 000 sperm mL^{-1}.

Although little research has been conducted on the effect of egg concentrations in abalone fertilisation, it is important in other marine molluscs (Crawford *et al.* 1986; Rampersad *et al.* 1994; O'Connor & Heasman 1995). When evaluating the influence of egg concentration on hatch-out rate in this study, it was concluded that concentrations between 10 and 125 eggs mL^{-1} resulted in the highest hatch-out rates when eggs were fertilised in standard 250 mL Erlenmeyer flasks.

Egg viability after spawning also varies between species, with a progressive decline in fertilisation potential relative to time post-spawning (Encena *et al.* 1998; Moss 1998; Babcock & Keesing 1999; Baker & Tyler 2001; Riffell *et al.* 2002; Grubert 2005; Zhang 2008). In this study, *H. midae* eggs remained viable for up to 100 minutes post-spawning, but after this period, hatch-out rates decreased linearly with egg age.

As it is not always possible to optimise these methods in a hatchery system, the use of the fertilisation model developed in this study could potentially be used as a basis to predict the outcome of fertilisation experiments. For this reason, the effect of fertilisation volume was also evaluated in relation to other factors measured, as this will enable hatcheries to translate result found in this study to the larger volumes of the hatchery systems.

6.2.1. Recommendations and future studies

It is advised that gamete concentrations be kept in ratio of 50 000 sperm mL^{-1} to 10 eggs mL^{-1} in a volume of 200 mL FSW for optimal hatch-out rates. Future studies should verify these results by implementing the developed fertilisation protocol in commercial hatchery systems. It should also be kept in mind that fertilisation should occur as soon as the eggs are liberated, as they will lose viability beyond 100 min post-spawning.

6.3. The use of a toxicity assay to determine larval competence in *Haliotis midae*

For commercial hatcheries, it would be advantageous to determine larval competence before settlement, as settlement success after hatch-out decreases from approximately 80% to 50%. In this study, a test was developed to evaluate larval competence before settlement, enabling hatcheries to predict settlement success.

As dimethyl sulfoxide (Me_2SO) is non-toxic to humans, it was used as chemical stressor to determine larval competence in *H. midae*. The general critical point at which 50% of *H. midae* larvae exhibited morphological abnormalities, i.e. the effective concentration (EC_{50}), was found to be at a concentration of 0.7% Me_2SO. Larvae with higher resistance against the toxic effects of Me_2SO will exceed this critical point by showing less morphological abnormalities when incubated for 24h in Me_2SO.

6.3.1. Recommendations and future studies

When fertilising an egg batch, the toxicity assay should be conducted concurrently in the laboratory. Twenty four hours post-fertilisation, larval hatch-out and the degree of abnormalities must be recorded. From the results obtained at this stage, it would be possible to predict the larval competence and potential settlement rate before continuing with settlement.

Other applications of this toxicity assay could be the evaluation of water quality or the effectiveness of sterilisation protocols. Another application might be the development of a cryopreservation method for

H. midae larvae and gametes. The toxicity of cryoprotectants needs to be evaluated before the initialisation of a freezing protocol. In a previous study (Roux *et al*. 2008), the toxic effect of methanol (MET), polyethylene glycol (PEG), dimethyl sulfoxide (ME_2SO) and glycerol (GLY) on *H. midae* embryos was investigated. Results indicated that PEG and MET had the least toxic effect on *H. midae* embryos in concentrations ranging from 5% to 15%, while Me_2SO and GLY resulted in dead or abnormal embryos at concentrations above 15%. These results together with the technique developed in this study, could lead to the development of a cryopreservation protocol specifically for *H. midae* sperm and eggs.

Future work should include the evaluation of larval competence beyond 24 hours to determine the long-term effect of Me_2SO. Other toxicants might also be evaluated for their potential toxic effect on developing larvae.

6.4. Hybridisation of *Haliotis midae* and *H. spadicea*

Hybrids have been produced in various aquatic species, including abalone (*Haliotis discus discus* X *H. gigantean* X *H. madaka*), mussel (*Mytilus edulis* X *M. trossulus*), prawn (*Penaeus setiferus* X *P. vannamei*), sea urchin (*Strongylocentrotus nudus* X *S. intermedius* X *Anthocidaris crassispina*) and catfish (*Silurus glanis* X *S. aristotelis*) (Misamore & Browdy 1997; Paschos *et al.* 2004; Toro *et al.* 2004; Ding *et al.* 2007; Ahmed *et al.* 2008). When broodstock with a high reproductive efficiency have been identified by means of the various criteria discussed in this study, the hybridisation potential of selected broodstock with other indigenous species can be investigated. Heterosis can potentially enhance various traits of the paternal and maternal stock, producing hybrid offspring with increased growth rate, improved disease resistance, improved meat quality, or higher environmental tolerance; thus overall contributing to the commercial viability of abalone cultivation (Paschos *et al.* 2004; Ding *et al.* 2007).

In this study, the hybridisation potential between *H. midae* and *H. spadicea* was investigated by using a needle biopsy technique. Using testicular spermatozoa aspirated from *H. spadicea*, spawned *H. midae* eggs were fertilised and viable larvae produced. Quantitative real-time PCR and high-resolution melt-curve (HRMC) analysis were performed to genetically verify the status of *H. midae* X *H. spadicea* hybrid larvae (Slabbert 2010). Although interspecific hatch-out rates (3.9±0.9%) were significantly lower ($p < 0.05$) than that of intraspecific fertilisations (66.9±8.5%) when using biopsied sperm, this study proved that hybridisation between these two species was possible.

6.4.1. Recommendations and future studies

Further studies should investigate methods to increase the yield of spermatozoa from testicular biopsies. In doing so, optimal spermatozoa concentrations for fertilisation can be established. Techniques should also be developed to extract viable eggs from female abalone. This will assist in obtaining eggs from females, which are not yet conditioned to spawn. After these initial parameters are optimised, hybrid larvae can be evaluated for potential hybrid vigour. When beneficial characteristics of hybrids are identified, cultivation of sterile hybrids could prove to enhance current abalone cultivation in South Africa.

6.5. Main conclusions

The main conclusions from this study are that:

- oogenesis can be characterised by nine main development stages;
- spermatogenesis can be characterised by five main development stages;
- cultivated *H. midae* can be selected for breeding protocols from 24 months of age;
- optimal fertilisation can be achieved by fertilising eggs at a ratio of 1:50 in 200 mL FSW;
- eggs should be fertilised between five to 100 minutes post-spawning;
- competent larvae can be identified by their enhanced tolerance of 0.7% Me_2SO;
- fertilisation can be achieved by using biopsied sperm; and
- hybridisation between *H. midae* and *H. spadicea* is possible when using biopsied sperm;

During the course of this study the following aspects were observed; egg size between individual females as well as between spawnings did not vary; egg lipid content and larval competence displayed an inverse relationship; larval competence increased when egg carotenoid levels increased; egg colour varied between females but remained similar for individual females between spawnings; egg colour and carotenoid content seemed to be related; and sperm competition between males was observed during mass spawnings. These aspects should be further investigated in future studies on the reproduction of abalone.

The current study contributes significantly to the current knowledge of abalone aquaculture and specifically the reproduction of *H. midae*. The results obtained from this study have the capacity to be of immediate application and benefit to the abalone industry and the techniques developed could be transferred to other species of abalone and other related molluscs.

6.6. References

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