

Factors influencing the larval settlement of abalone (Gastropoda: *Haliotis midae*): considerations for stock enhancement and ranching

Submitted by

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Supervised by Prof. NG Vine

Declaration statement

I, **Ziyanda Mzozo**, declare that this research thesis is my work, and it has not been submitted in any previous application for a degree. Information derived from the published or unpublished work of others has been acknowledged in the text, and a list of references is given. The data presented in this thesis was obtained in the experiment series that I carried out, except for ocean quadrant survey data, which the commercial divers collected on my behalf. This research followed the University of Hare guidelines and policies on academic plagiarism.

Signature:



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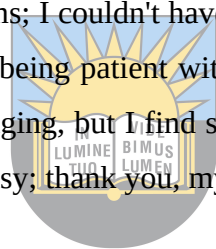
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Abstract

The South African wild abalone, *Haliotis midae*, is essential for critical commercial fisheries; however, the population has declined significantly over the past four decades due to overfishing and poaching. This thesis was developed in response to the decline of wild abalone stocks and the high costs associated with seeding juvenile abalone to help improve the wild population. Concerns over these costs have led to increased interest in seeding abalone larvae, as millions can be produced in a basic hatchery, resulting in lower expenses.

A series of hatchery-based and ocean-seeding experiments were conducted to establish baseline methods and make recommendations for enhancing or ranching competent *H. midae* larvae.

In one hatchery experiment, the impact of temperature on the development and settlement of *H. midae* larvae was assessed. The results indicated that both larval development and settlement improved with every 2°C increase in temperature within the range of 14°C to 22°C. Therefore, it is recommended that larvae be seeded at temperatures between 20°C and 22°C to potentially increase settlement rates and shorten the settlement period.

The study also investigated the effectiveness of chemical and biological cues in improving the settlement and metamorphosis of competent *H. midae* larvae before their release into the ocean. It was found that settlement cues were only effective when water flow was turned off, and the highest settlement rates occurred with a combination of potassium chloride (10 mM KCl) and biological cues from *Nitzschia sp.* diatoms. However, all treatments exhibited lower settlement responses when water flow was present. This suggests that chemical cues may not be effective if larvae do not receive them until they are fully settled. Continuous exposure to an inducer until metamorphosis, achievable in a hatchery but not in the ocean, did enhance settlement. This finding highlights the potential for application within the hatchery environment.

Tagging released larvae is fundamental for evaluating the success of a seeding program. In a hatchery experiment, bright and persistent epifluorescence marks were successfully induced in *H. midae* shells using calcein (25 mg.L⁻¹ for 24 hours) without negatively affecting the survival or settlement of the larvae post-immersion.

To evaluate the viability of using abalone seeding as a tool for ranching and stock enhancement, competent, fluorescent-stained *H. midae* larvae were released at three sites less than two meters apart in Noordhoek (Gqeberha) along the Eastern Cape coastline. Although the density of the released larvae ($2.22 \pm 1.09 \text{ m}^{-2}$) was lower than that of wild abalone ($37.96 \pm 7.45 \text{ m}^{-2}$), the growth rate of the released abalone was not hindered. Results from this pilot study demonstrate that releasing hatchery-reared *H. midae* larvae has the potential to support abalone ranching and enhance the wild abalone population along the Eastern Cape coastline. While the technique requires further refinement, these findings provide a foundational methodology for implementing the release of *H. midae* larvae in this region.

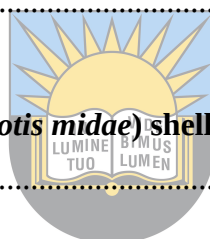


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CHAPTER 1

Literature Review

1.1 Biology of abalone

Abalone are marine snails that inhabit the lower intertidal zone and sublittoral fringe of rocky shores (Freeman 2001, Stephens et al. 2006, Leighton 2008, Cloete 2009, van der Merwe 2009). They have adapted to these conditions by developing a muscular foot that attaches securely to the rock substrate, preventing them from being swept away by wave action, as well as a strong shell that protects their body tissue (Heasman & Savva 2007). The muscular foot is also used for locomotion (Madikiza 2012). Abalone are widely distributed across tropical and temperate coastlines (Freeman 2001), with the South African abalone, *Haliotis midae*, found along the South African coastline from St. Helena Bay on the West Coast to just north of Port St. Johns on the East Coast (DAFF 2017).

Abalone are slow-growing and late-maturing species, with their growth (Pedroso 2017) and reproductive cycles regulated by temperature (Britz et al. 1997; Leighton 2008). Optimal temperatures for each species influence reproductive hormones (Grubert & Ritar 2005), leading tropical species to spawn year-round, while temperate species typically have distinct spawning seasons. For example, *H. midae* experiences peak spawning activity between April and June (Wood 1993). Nutrient availability also impacts the reproductive cycle of abalone by affecting gonad development. Al Rashidi (2008) noted that *Haliotid mariae* reproduce in the months following the monsoon. The moon influences upwellings that increase nutrient concentration in the water (Al Rashidi 2008), highlighting the importance of nutrients in the reproduction cycle of these species.

Abalone are broadcast spawner (Stephens et al. 2006; Heasman & Savva 2007; Leighton 2008). A female abalone can release millions of eggs into the water, which are then fertilized by sperm from male abalone (Leighton 2008). Inshore wave currents facilitate high fertilization success (Stephens et al. 2006; Heasman & Savva 2007). The fertilized eggs hatch into active swimming

larvae called lecithotrophic larvae (non-feeding organisms) (Stephens et al. 2006; Leighton 2008). These larvae are pelagic and can be passively transported by currents, allowing for wide dispersion (Prince et al. 1987).

However, literature presents contradictory views regarding the processes that occur before larvae settle on a substrate.: Prince et al. (1987) reported short-distance dispersal of abalone larvae, *Haliotis rubra*, which was associated with the densities of spawning adults. Similar findings were obtained by McShane (1992), for *Haliotis iris* larvae, which settled close to adults. Shephard et al. (1992) reported that bays generate eddy systems with countercurrents and stagnation zones, which concentrate plankton to the background levels. Alldredge and Hammer (1980) reported the downward vertical component from the reverse current assists in concentrating larvae at the surface and while flood current help dispersed larvae inshore. This dispersal mode benefits abalone larvae by increases the chances of settlement and survival for abalone in proximity to their parents' locations (Miyake et al. 2017).

On the other hand, Shepherd et al. (1992) reported long-distance dispersal for *Haliotis laevis*, observed no relationship between recruitment and adults. The authors concluded that the wave-transported larvae tend to settle on a substrate close to the bottom, with shore topography creating eddies and stagnation zones. Tegner (1992) reported similar results: there was no relationship between the adults and juveniles of *Haliotis fulgens*. This longer dispersal mode can reduce the chances of abalone larval settlement if the current transports the larvae to sandy area where they cannot settle. Stephens et al. (2006) reported a dispersal mode – both short and long dispersal. When water flow is low, *H. iris* larvae tend to settle near their parent due to low wind force affects ocean current. Conversely, strong wind creates strong current that disperse larvae inshore away from their parents. Chick (2010a) reported that water flow influences the settlement of larvae (*H. rubra*). McCarroll et al. (2021) supported the dual dispersal mode, indicating that under low energy-forcing wind conditions, particles remaining near the coast while under high energy-forcing winds could disperse particles offshore.

During their pelagic phase, abalone larvae undergo a series of physical and physiological developmental stages (Genade et al. 1988; Leighton 2008). The biological and adaptive behavior of each species determines the duration of their pelagic phase (Moss 1999). After about a week—depending on water temperature—larvae reach the settlement stage, at which point they search for a suitable substrate to attach and grow (Maxwell 2014). Settlement involves transitioning from a swimming larva to a crawling form; during which the larvae lose

their velum and attach to the substrate (Roberts & Lapworth 2001, Leighton 2008, Maxwell 2014). Metamorphosis is an irreversible process in which the mantle membrane separates from the larval shell and moves to the shell edge, after which juvenile shell growth commences (Leighton 2008).

Inducing larval settlement in abalone

Larval settlement and metamorphosis in abalone are complex processes, and the factors influencing them—both internal (endogenous) and external (exogenous)—are often not well understood (Tebben et al., 2015). One key internal factor that prompts settlement is the biological development of abalone larvae. The larvae can only settle after reaching a specific morphological stage, which is characterized by the presence of nine rows of teeth on the radula, as well as certain behavioral developments such as crawling and exploring the surface with their foot (Moss and Tong, 1992b).

The external factors that typically induce larval settlement are known as settlement cues. These cues are primary stimuli that initiate the settlement and metamorphosis of competent larvae in many marine mollusks (Li et al., 2006). Settlement cues come from substrates that provide newly settled juveniles with both nutrition and refuge (Yool et al., 1986). These substrate cues are transmitted to the nervous system of the abalone larvae, triggering the processes of settlement and metamorphosis (Yool et al., 1986).

Settlement cues can originate from various sources in the marine environment; they may be waterborne, associated with the surfaces of substrates, or linked to individuals of the same species (Morse, 1991). All these cues convey important information about the habitat available post-settlement (Tebben et al., 2015). Research indicates a relationship between endogenous and exogenous factors in abalone larval settlement, demonstrating that larvae respond to settlement cues only when they have fully developed (Morse, 1991).

Habitat and feeding

Abalone larvae require specific habitat conditions for settlement, with consolidated rock or boulder reef areas indicating suitable environments. Depth and rugosity also play crucial roles in habitat selection. In contrast, sandy substrates are a negative indicator of suitable habitats for abalone (Moriarty 2019). After settling, abalone larvae metamorphose into juveniles and typically settle on consolidated rocks or boulders that are encrusted with coralline algae (Day & Branch 2000).

Once settled, newly transformed juveniles graze primarily on diatoms. These juveniles are highly cryptic, necessitating habitats rich in crevices and hiding places (Wood 1993). They often share a commensal relationship with sea urchins, seeking refuge from predators under the spines of the urchins (Day & Branch 2000).

Both juvenile and adult abalone are herbivorous, feeding on both attached and drifting algae (Henry 1995; Heasman & Savva, 2007). The detached seaweed, commonly referred to as drift, serves as their primary food source (Heasman & Savva, 2007). The South African species *H. midae* is particularly noted for its deep red shell coloration, which is primarily derived from its diet of red algae

1.2 A history of the South Africa abalone fishery

Abalone has been harvested from the wild for many decades globally and is frequently exported to Asia, with Hong Kong, Japan, and Singapore being the leading importers (Cook 2023). In these countries, abalone is regarded as a delicacy, particularly in metropolitan areas, and China is the largest consumer of this seafood (Cook 2023). It is traditionally served at weddings and on New Year's Eve in various Asian cultures (Sales & Britz 2001). Abalone is marketed in multiple forms, including canned or dried meat from the muscular foot or as live abalone, while the shells are often used for making jewelry (Cloete 2009, DAFF 2017; Sales & Britz 2001).

The growing demand for abalone products in Asia led to the establishment of commercial abalone fishing in South Africa during the early 1950s (Britz et al. 2009; Henry 1995). Initially, abalone was fished for commercial, recreational, and decorative purposes without regulation (Madikiza 2012). However, by the early 1970s, catch levels began to decline due to this unregulated fishing. In the early 1990s, an increase in recreational fishing alongside a significant spike in illegal fishing activities further contributed to dwindling wild stocks. Consequently, recreational fisheries were closed in 2003 (South African Government Gazette 2010; Madikiza 2012; DFFE 2023).

In 2003, the South African government introduced long-term fishing rights and Territorial Use Rights in Fisheries (TURFs) to co-manage resources and enhance compliance with fishing regulations. However, illegal fishing activities continued to fluctuate in various locations, often shifting in response to resource availability and law enforcement presence (DFFE 2023). Despite the introduction of TURFs, illegal fishing remained prevalent, and wild abalone stocks continued to decline. In 2008, the government imposed a ban on diving in selected areas and closed the commercial fishery in February of that year (DFFE 2023). The fishery was reopened

in July 2010 but aimed for a 15% annual reduction in illegal fishing, a target that has not yet been met (DFFE 2023). Recently, the total legal commercial catch has been recorded at 53 tonne (Fig. 1.1), indicating severe depletion of wild stocks and ongoing heavy pressure from illegal fishing (DFFE 2023).

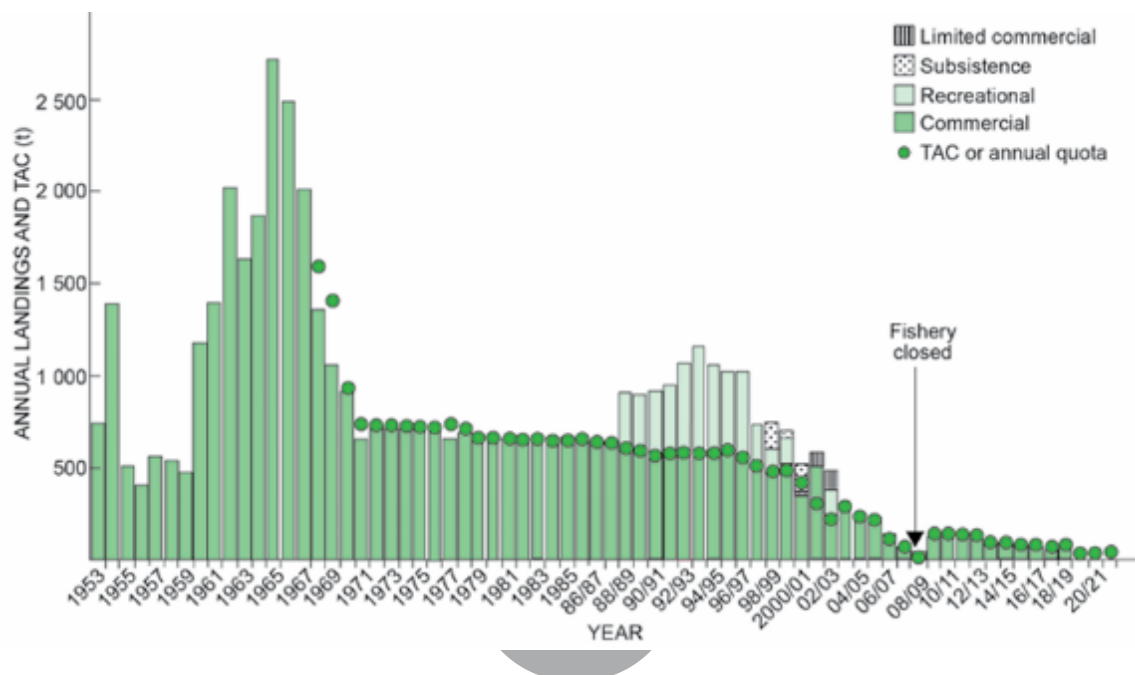


Figure 1.1 The recorded legal annual landing and the total allowable catch of South African abalone from 1953 to 2021 (DFFE 2023)

The dramatic increase in the global human population over the past four decades has contributed to a doubling of seafood consumption (Stickney 2005), further amplify the decline of abalone fisheries due to over-exploitation, lack of spatial management, and various diseases affecting populations worldwide. Countries such as the United States (California) and Canada (British Columbia) have completely decommercialized their abalone fisheries and are on the verge of collapse (Cook 2023). While wild harvests are still carried out in South Africa, New Zealand, Australia, Mexico, and Japan, all have experienced significant declines (Cook 2023). Globally, the total wild abalone harvest has plummeted from nearly 19,720 metric tonne (mt) in the 1970s to less than 4,510 mt in 2021 (Cook 2023).

The reasons for the collapse of abalone fisheries vary by country. In Australia, a viral outbreak known as abalone viral ganglioneuritis (AVG) has significantly affected wild stocks, causing tissue necrosis and lesions (Mayfield et al. 2011). In the USA, overfishing has led to a rapid

decline in fisheries (Cook 2016). Japan faces challenges from overfishing, coastal development, and increased sedimentation (Uchino et al. 2004), while New Zealand's decline is also attributed to overfishing (Roberts et al. 2007). In South Africa, illegal fishing, or poaching, is primarily responsible for abalone fisheries' decline. This rise in poaching began in the 1990s following the depreciation of the South African rand against the US dollar, which significantly increased the price premium for abalone and created new trade opportunities with Asia (Raemaekers et al. 2011). The high market value, coupled with the accessibility of abalone in shallow intertidal areas, fueled rampant poaching (De Waal 2002). Illegal fishing practices hinder the recovery of wild abalone stocks, as poachers often ignore the minimum legal size (11.4 cm, standard body length) for harvesting, collecting immature abalones before they can reproduce (DFFE 2023). A recent survey indicated that 65% of dried South African abalone sold in Hong Kong was illegally sourced, 33% came from legal aquaculture, and only 2% was from legal fisheries (Cook 2023). Despite South Africa often being labeled the main supplier of illegal abalone, the complexities of the situation highlight broader challenges facing abalone fisheries globally.

1.3 Abalone farming

Abalone is one of the most expensive seafood products worldwide (Sales & Britz 2001, Cloete 2009, Cook 2016, FAO 2018). The rising commercial value of abalone has triggered advancements in abalone farming technology (Sales & Britz 2001, De Waal 2002, Cook 2014). Since the late 1950s, aquaculture has served as a bridge between overfished abalone fisheries and the growing global demand, alleviating fishing pressures on wild abalone stocks (Sales & Britz 2001, Pang et al. 2006; Troell et al. 2006). This practice has produced a reliable, year-round supply of high-quality abalone (Freeman 2001a, Troell et al. 2006). The rapid expansion of the abalone aquaculture sector can be attributed to improvements in culture techniques, the development of formulated feeds, and better management and understanding of diseases related to aquaculture (van der Merwe 2009). Abalone farming began in Japan and China during the late 1950s and early 1960s (Hamasaki & Kitada 2008), with significant increases in farmed abalone occurring from the 1970s onward (Cook 2014). Abalone aquaculture has expanded significantly and is now practiced in various countries, including the USA, Mexico, South Africa, Chile, Australia, New Zealand, Korea, and Taiwan (Troell et al. 2006, Cloete 2009). It is one of the fastest developing industries worldwide (Pang et al. 2006, Cook 2016, FAO 2018), with over 95% of abalone in the export market coming from aquaculture (Troell et al. 2006,



Cook 2016). As of 2023, China is the largest producer of farmed abalone, with over 217,000 tonne, followed by Korea, which produces over 205,000 tonne (Cook 2023).

In response to declining yields from wild fisheries (Troell et al. 2006), a focused research and development effort to establish commercial abalone farming in South Africa began in 1990 (Sales & Britz 2001). This initiative combined technology transfer from other countries with local innovation (Sales & Britz 2001). South African abalone aquaculture systems were designed with land-based facilities, including independent hatcheries and grow-out unit (Troell et al. 2006, DAFF 2017), as well as sea cage farming (DFFE 2021). The South African industry has developed rapidly over the years (Fig. 1.2) compared to countries with longer histories of abalone farming (Godfrey 2003, Troell et al. 2006). By 2003, South Africa's abalone aquaculture had grown to over 500 tonnes; by 2013, this figure had increased to approximately 1,400 tons (DAFF 2017). In 2021, South Africa produced 2,664 tons, making it the third-largest farmed abalone producer worldwide (Cook 2023). Abalone is now the second-largest sub-sector, contributing 43% to the country's total production (DFFE 2021).

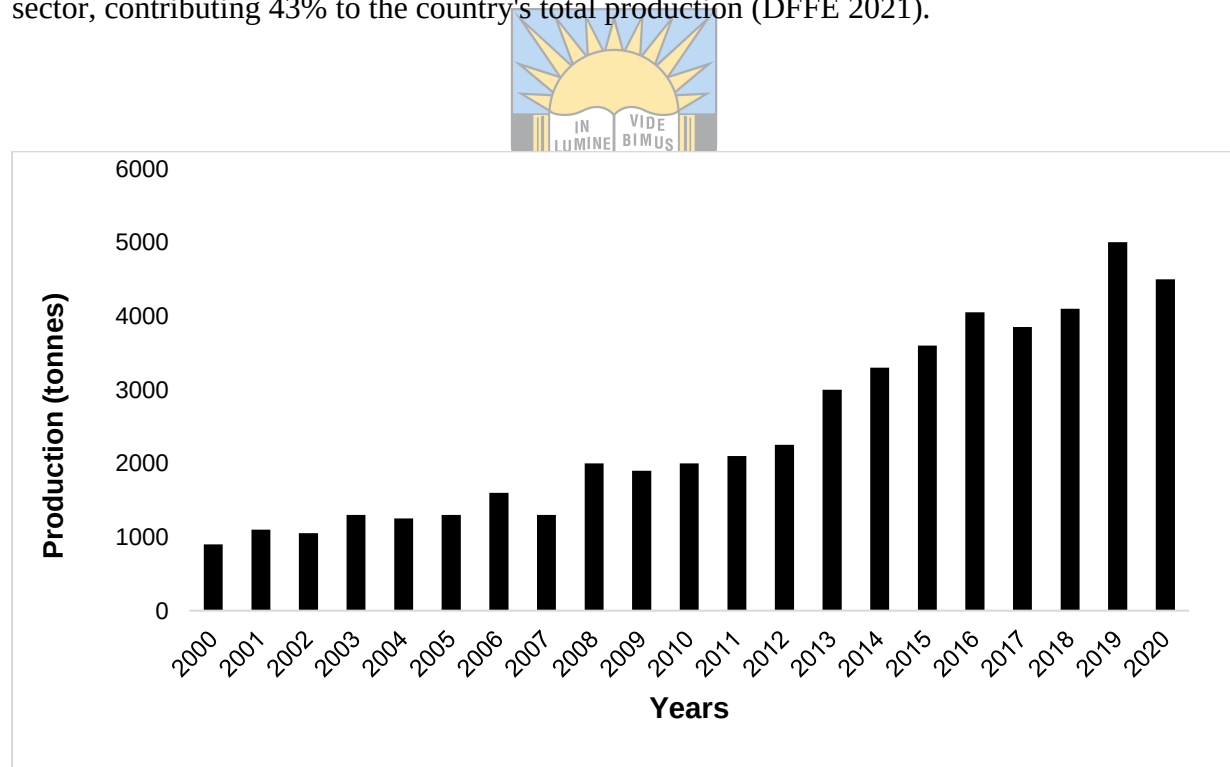


Figure 1.2 Farmed abalone production in South Africa from 2000 - 2020 (DFFE, 2021)

Farmed South African abalone is exported as live, frozen, dried (preserved) and canned to Asian countries (such as Hong Kong, China, Japan, Singapore, Malaysia, Vietnam, and South

Korea), as well as to Australia and the United States of America (DFFE 2021). The price of abalone in the global market depends on factors such as species, culture, country of origin, quality of meat, and size (Cook 2016), with South African abalone regarded as among the best in the world (Prof. Britz, Rhodes University, pers. comm.).

Despite the rapid growth of abalone farming in South Africa, land-based operations face significant challenges. High fixed capital investments are required for land-based farming (De Waal 2002, Godfrey 2003, Troell et al. 2006, Cloete 2009). Most South African abalone farms utilize a flow-through aquaculture system, where seawater is pumped from the ocean into the farm, filtered into the abalone tanks and then returned to the sea. Some farms recycle part of their effluent water through seaweed tanks to feed the abalone. Operating the large pumps needed for this system incurs substantial electricity costs, which can account for up to 24% of the farm's operational expenses. Additionally, abalone farming is labor-intensive, with wages, salaries, and protective gear making up another 72% of operational costs. Formulated feeds contribute about 0.4%, and other consumables add approximately 2.6% to overall operational expenses (Abagold 2021). Various biological factors, such as the slow growth rate of abalone influenced by water temperature, food type and availability, water quality, and management practices (including grading and stocking densities) also affect production costs. Poor management of these factors can lead to increased operational costs. Nevertheless, the rising demand for South African abalone in East Asia has driven significant growth in abalone farming within the country.

1.4 Abalone ranching and stock enhancement

Owing to the high capital and operational costs associated with land-based abalone aquaculture, farmers have been exploring the potential for abalone ranching (De Waal 2002, Witte 2017). Abalone ranching involves releasing cultured animals into the ocean to be harvested later (Bell et al. 2008). This approach offers a more cost-effective production method compared to traditional land-based farms (DFFE 2021). Ranched abalone grow at no expense to the farmer, effectively separating abalone farms from costs related to electricity, labor, and feed (Troell et al. 2006). Additionally, abalone ranching can contribute to the recovery of wild abalone stocks; if abalone is not harvested until it reaches the breeding size of 400–600 g after 5–7 years, it can help replenish wild populations (Bell et al. 2008). While stock enhancement specifically refers to releasing cultured juveniles into the wild to boost the natural supply and optimize harvests, it engages the same technical capabilities as abalone ranching (Bell et al. 2008).

Abalone ranching has been practiced in Japan since the late 1960s. Although these releases have not been able to sustain total production on a national scale, local studies suggest that hatchery releases may enhance total production in specific areas (Hamasaki & Kitada 2008). Similar experimental stock enhancement programs have been undertaken in Australia (McCormick et al. 1994, Preece et al. 1997, Goodsell et al. 2006, Chick et al. 2013), the USA (Hansen & Gosselin 2016), and New Zealand (Tong et al. 1987, Roberts et al. 2007). While results have varied, none of these programs have been fully commercialized.

In South Africa, abalone ranching has been tested experimentally to evaluate its potential for rebuilding depleted abalone populations or creating new fisheries. The first juvenile abalone seeding took place in Port Nolloth, Western Cape, in an area beyond the species' natural range. Sweijd et al. (1998) reported a 30% survival rate of juvenile *H. midae* after six months, with abalone taking 3.5 to 6 years to reach market size. Godfrey (2003) also conducted an abalone seeding experiment near Port Elizabeth, Eastern Cape, in an area affected by overfishing, which indicated commercial potential. More recently, Witte (2017) performed a juvenile abalone ranching experiment at Cape Recife, supporting Godfrey's (2003) conclusion regarding the area's potential. Currently, there are three commercial-scale abalone ranching operations in South Africa (DFFE 2021).

1.4.1 Seeding using juvenile abalone

Most experimental trials for abalone ranching and stock enhancement have focused on using hatchery-reared juveniles (McCormick et al. 1994, Godfrey 2003, Goodsell et al. 2006, Chick et al. 2013, Witte 2017). It is commonly believed that rearing juveniles for two to three years increases their chances of survival by reducing susceptibility to predation (Hansen & Gosselin 2016). While the results of juvenile seeding studies vary, all suggest that releasing juvenile abalone might help rebuild or sustain wild populations. However, these studies have also documented high mortality rates and low recapture rates. Hansen and Gosselin (2016) estimated that 66% of *Haliotis kamtschatkana* mortalities occurred within two weeks post-seeding, while similar wild species showed less than 1% mortality during the same timeframe. In the USA, fewer than 1% of *H. rufescens* juveniles survived to harvestable size despite ongoing seeding (Rogers-Bennett et al. 2016). In Australia, the survival rate for *H. laevigata* juveniles after nine months ranged from 0% to 57% (Dixon et al. 2006), and in South Africa, the survival rate of *H. midae* varied between 4% and 27% after 666 days (Witte 2017).

The high mortality rate observed in seeded juvenile abalone is attributed to several factors, with predation being the most significant (Hansen & Gosselin 2016). Juvenile abalone from hatcheries may behave differently than their wild counterparts, making them more vulnerable to predators and leading to higher mortality rates in the wild (McCormick et al. 1994). For example, hatchery-produced *H. kamtschatkana* were observed to take longer to evade predators and moved more slowly into hiding places due to a lack of ecological experience (Hansen & Gosselin 2016). The high mortality rates could also be related to stress caused by multiple handling events during the transfer from farm to ocean (Chick 2010a).

Although abalone ranching has been seen as a potential cost-reducing tool for fisheries, its economic viability was considered marginal in the early 1990s. The increase in poaching in South Africa during the late 1990s led to a decline in wild abalone stock, led to abalone ranching being investigated to rehabilitate wild stock damaged by poaching. The South African government will award harvesting rights to a portion of the resource to the companies or communities that invest in the resource through reseedling of abalone and providing private security. Abalone ranching is now being investigated to gain an understanding of the technologies and improve the knowledge that enables economically viable release of abalone into the wild. The method is currently being implemented around South Africa.

1.4.2 Seeding using abalone larvae

Concerns regarding the high mortality rates and costs associated with the seeding of juvenile abalone have led to increased interest in using abalone larvae for seeding. Various seeding methods have been investigated, including the mass release of veliger larvae (Tong et al. 1987, Preece et al. 1997, Heasman et al. 2004, Chick 2010b, Mills-Orcutt et al. 2020). Utilizing abalone larvae for seeding could significantly reduce costs, as millions can be produced in a basic hatchery (Preece et al. 1997). Furthermore, the rearing time in the hatchery is shortened from approximately nine months to as little as four to ten days, depending on the species and the rearing temperature.

Introducing abalone larvae at an earlier stage may also enhance their ability to adapt to environmental conditions in the ocean (Hansen & Gosselin 2016). This is particularly advantageous given the brief veliger phase of haliotid species (which lasts four to ten days), potentially increasing seeding efficiency by decreasing the likelihood of hatchery acclimation and adaptation (Mills-Orcutt et al. 2020). Keeping larvae in the hatchery until just before they settle may help reduce the dispersal period, significantly improving their chances of settling in

the immediate area and enhancing survival rates in the wild (Hansen & Gosselin 2016, Mills-Orcutt et al. 2020).

However, the effectiveness of this restoration tool has not been extensively studied compared to seeding juveniles. Only a limited number of studies (Tong et al. 1987, Shepherd et al. 1992, Preece et al. 1997, Heasman et al. 2004, Chick 2010b, Mills-Orcutt et al. 2020) have been conducted on different species at an experimental scale. Results have shown significant variation in survival rates depending on the species and the methods used, indicating that strategies for seeding larvae are both site- and species-specific.

Abalone ranching is subject to specific regulations; for instance, if the stock moves out of its designated ranching area, the right holder has no entitlement to recover it (South African Government Gazette 2010). A critical concern with seeding early-stage abalone larvae is their potential to disperse beyond the intended seeding area, which could lead to the failure of abalone ranching efforts.

Prince et al. (1987) found that the dispersal capabilities of haliotid larvae are constrained only by the time available before they settle. Consequently, research on the viability of seeding abalone larvae has primarily focused on developing different out-planting strategies to minimize larval dispersal. For instance, Tong et al. (1987) in New Zealand seeded older, competent *H. iris* larvae at 13 days old, even though they were ready to settle at nine days. Authors reported that older larvae settled more quickly when in proximity to suitable substrates. Preece et al. (1997) employed pyramid-shaped nylon mesh tents to retain larvae released under their coverage in Australia. This tent structure was typically removed after one day, although tents remained for seven days in areas with rough seas. Similarly, in the United States, Hansen (2006) and Mills-Orcutt et al. (2020) seeded *H. kamtschatkana* using tents to retain larvae for two days post-seeding. Another strategy tested to reduce the dispersal time of abalone larvae after seeding involved the use of chemical cues. Hansen (2006) and Mills-Orcutt et al. (2020) exposed *H. kamtschatkana* to Gamma-amino-n-butyric acid (GABA) for 30 minutes prior to seeding in order to stimulate settlement and metamorphosis. Both seeding events were considered successful, with Hansen (2006) reporting that seeding abalone larvae was generally more effective than seeding juveniles.

Although the technique for seeding abalone larvae has been tested in various studies from different regions, it has yet to be evaluated along the South African coastline. Previous experiments were conducted in calm seas, which do not represent the conditions of strong

ocean currents. The Eastern Cape coastline of South Africa is highly exposed to the robust Agulhas currents, which flow from ENE to WSW and cause upwelling and downwelling events driven by the wind and the meanders of the Agulhas Current (Goschen et al. 2012). While McCarroll et al. (2021) suggested an optimal release point to maximize the residency of *H. midae* based on prevailing wind conditions, more research is needed to assess the ability of *H. midae* larvae to endure these conditions and to successfully settle and metamorphose on the reef.

Despite concerns about the dispersal of abalone larvae during ocean seeding, one of the main issues with using abalone larvae for seeding is the unpredictability of settlement (Gapasin & Polohan 2004). Both the settlement and metamorphosis stages of abalone larvae are critical in their life cycle (Yu et al. 2010). Low post-larval survival rates are often linked to inadequate larval settlement and metamorphosis (Gapasin & Polohan 2004, Li et al. 2006). Li et al. (2006) found that only a maximum of 10% of *Haliotis diversicolor* larvae transitioned from planktonic veliger to benthic juveniles, with the remainder dying under controlled hatchery conditions

1.5 Factors influencing larval settlement

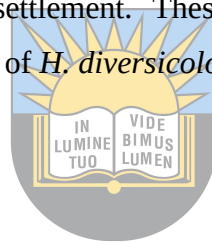
1.5.1 Chemical settlement cues

Coralline algae

Coralline algae is crucial in the life cycles of various marine invertebrates, as it induce both the settlement and metamorphosis of larvae (Roberts & Lapworth 2001, Huggett et al. 2005a, Chick 2010a). Crustose coralline algae (CCA) naturally occur on reef surfaces, producing chemical cues in the form of small peptides associated with proteins on their surfaces (Hadfield & Paul 2001). Abalone larvae can sense and recognize these chemical compounds, specifically phycobiliproteins found on the coralline algae's surface, prompting their settlement. Different species of abalone larvae respond variably to different species of coralline algae, indicating a species-specific relationship coralline algae species (Robert 2001). While the physical characteristics of CCA, such as texture and morphology, influence larval settlement, Daume et al. (1999a) reported that these physical traits are not the primary factors inducing settlement. In hatcheries, chemicals extracted and purified from coralline algae or synthetic chemicals that mimic CCA are often used to stimulate settlement. These waterborne cues derived from CCA are known to induce larval settlement (Morse 1991).

γ-Aminobutyric Acid (GABA)

Although CCA is a significant inducer of abalone larvae settlement in the ocean, similar techniques cannot be effectively applied in abalone hatcheries due to the challenges of culturing or growing crustose coralline algae in land-based facilities. Water-soluble extracts from coralline algae can induce settlement in laboratory settings (Hadfield & Paul 2001). Consequently, commercially sourced chemicals, such as γ -aminobutyric acid (GABA), which mimic the phycobiliproteins of CCA, are primarily utilized in abalone hatcheries or laboratory studies. The external chemical signal of GABA in the water translates into a nerve impulse, triggering responses in abalone larvae, such as cessation of swimming (where the veliger "falls" out of the solution), foot exploration behavior, and metamorphic changes (metamorphosis) (Robert 2001). Different concentrations of GABA can affect larval responses differently. A study by Bryan and Qian (1998) found that a high concentration of GABA (10^{-3} M) resulted in less than 5% settlement after 36 hours for *H. diversicolor*, compared to a low concentration (10^{-6} M) that induced over 40% settlement. These results suggest that while GABA significantly influences the settlement of *H. diversicolor*, high concentrations can be toxic and lead to increased mortality.



Potassium chloride (KCl)

Potassium chloride is another widely used chemical in hatcheries, as an increase in K^+ concentration in seawater has been shown to promote settlement and metamorphosis in marine invertebrates (Yool et al. 1986). Potassium ions can influence cell membrane potentials, activating sensory or inducing pathways (Yool et al. 1986). Several studies have used the technique of increasing external K^+ concentrations to induce settlement in various abalone species (Bryan & Qian 1998, Li et al. 2006, Yu et al. 2010). While Yool et al. (1987) initially suggested that raising K^+ concentrations would enhance settlement, there exists an optimum level beyond which high chemical concentrations are toxic to abalone larvae, resulting in higher mortality rates (Bryan & Qian 1998, Li et al. 2006, Yu et al. 2010). For instance, a study by Yu et al. (2010) demonstrated that 70 mM KCl resulted in low settlement rates (0-21%) for *H. diversicolor*, while 20 mM KCl yielded the highest settlement rates (24-56%). Furthermore, mortality levels for *H. diversicolor* were significantly higher at 70 mM KCl, reaching up to 100%, compared to 0-12% at 20 mM KCl.

Although the effects of GABA and KCl on settlement have been extensively researched, studies indicate that different species of *Haliotis* may respond differently to the same settlement

cue. Consequently, it is important to identify the optimal concentration of each inducer for each species, as their responses can vary considerably.

1.5.2 Biological cues

Natural microalgae and culture diatom biofilms

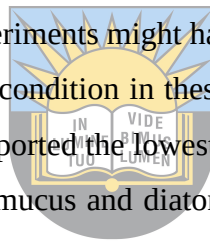
Natural diatoms develop in flowing seawater in response to light and colonize the aquatic substrate, forming a biofilm layer. Culture diatom biofilms consist of diatom species strains isolated and cultivated in the laboratory, allowed to proliferate on aquatic surfaces and produce a thin biofilm layer. Biofilms have long been used to induce larval settlement in abalone hatcheries worldwide (Leighton 2008).

Substrate-coated diatom layers have been reported to induce settlement and metamorphosis in abalone larvae (Bryan & Qian 1998, Daume et al. 1999b, Gallardo & Buen 2003, Gordon et al. 2004, Li et al. 2006). Diatoms excrete inducing compounds into the surrounding water, and these chemicals create gradients detectable by abalone larvae, inducing settlement (Gordon et al. 2004). However, different species of diatoms induce settlement differently. For example, Daume et al. (1999b) and Gordon et al. (2004) reported that *Navicula* spp induces higher settlement of *H. laevis* larvae than other diatoms. Gordon et al. (2004) reported that larval chemosensory response depended on the concentration of the inductive substance. This finding suggests that each strain of diatoms has a different inducing effect; hence, settlement success may differ. However, combining strong inducing diatoms with the less effective inducing diatoms may reduce the overall settlement percentage. For example, in abalone hatcheries, natural diatoms from the incoming seawater are used, which can be a combination of different strains of diatom (effective inducer and less effective inducer), which commonly results in a low settlement estimated at 1–10 % (Daume 2006, Li et al. 2006). Although *Navicula* might also be one of the natural diatoms used in hatcheries, other species strains might have hindered its inducing effect (Gordon et al. 2004). The author further reported that *Navicula* could effectively induce larval settlement as a single inducer or, in combination with another effective diatom inducer. Daume et al. (1999b) found that a combination of *Navicula* and *Amphora* was suitable for larval settlement.

Mucus substrate

Juvenile and adult abalone secrete foot mucus while crawling on the surface, the chemical composition of the mucus released by adults or juvenile abalone is based on their physiological

condition, indicating suitable or unsuitable conditions (Bryan & Qian 1998). Abalone larvae can determine the physiological condition of the surrounding environment based on the chemical compound secreted in conspecific mucus found on the substrate (Bryan & Qian 1998). McShane (1992) posited that abalone larvae might settle preferentially near conspecific adults because of the attractive presence of their mucus trails. Bacteria associated with the foot of the abalone may be primarily responsible for the induction of attachment (Bryan & Qian 1998). Pre-grazing by large conspecifics to develop a mucus substrate is another hatchery technique to improve larval settlement (Bryan & Qian 1998, Moss 1999, Gallardo & Buen 2003). Research by Bryan and Qian (1998), Gallardo and Buen (2003), Moss (1999) reports higher settlement of abalone larvae with a combination of mucus and diatoms than with mucus alone. Different compounds might be present in the foot mucus secreted by juvenile and adult abalone based on their physiological condition. Adults crawling on a preferred substratum may secrete compounds in their mucus, indicating favourable conditions, whereas stressed individuals may release compounds indicating unfavourable conditions. This suggests that the multi-handling of abalone during experiments might have caused stress, which led a to mucus secretion, indicating an unfavourable condition in these experiments. Bryan and Qian (1998) and Gallardo and Buen (2003) also reported the lowest level of post-larval survival on mucus alone compared to a combination of mucus and diatoms. One possible explanation could be that mucus alone does not provide sufficient nutrition for later post-larval stages (Takami et al. 1997).



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While extensive research has been conducting focusing on improving settlement and metamorphosis for commercial abalone species by investigating aspects such as chemical cues and biological cues, larval settlement and metamorphosis are often highly selective in that what works for one species may or may not work for another (Table 1.1)

Table 1.1 Induced settlement cues tested on abalone larvae

Abalone species	Developmental stage	Settlement cue	Study type	Outcome	Reference
<i>Haliotis tuberculata</i>	Competent (crawling and exploring)	Chemical (GABA), Biological (CCA, mucus, diatoms, <i>Ulvela lens</i>)	Laboratory	Larval settlement was highest on crustose coralline algae followed by <i>U. lens</i> . Settlement was very low on conspecific mucus and g-aminobutyric acid, varying between 1% and 2%. Settlement was very low and similar among diatom species.	De Viçose et al. (2010)
<i>Haliotis diversicolor</i>	Competent (crawling and exploring)	Chemical (GABA, KCl)	Laboratory	Highest settlement was in 10^{-4} M GABA (65%) in the first 24 hrs compared to 20mM KCl (20%). After 48 hours, 20 mM KCl (50%) was highest compared to 10^{-4} M GABA (40%). Metamorphosis was higher in 10^{-4} M GABA (40%), and there was no difference between 20 mM KCl and the control.	Yu et al. (2010)
<i>Haliotis asinina</i>	Competent (crawling and exploring)	Chemical (GABA, 5-AVA, GA, MSG)	Laboratory	5-AVA induced the highest settlement, followed by GABA, GA had the lowest settlement. The settlement did not differ between different concentrations (10^{-1} mM, 10^{-2} mM, 10^{-3} mM) of 5-AVA treatments.	Stewart et al. (2008)
<i>Haliotis rubra</i>	Competent (crawling and exploring)	Biological (CCA covered rock, diatoms)	Laboratory	CCA-coated rocks generally had more than double the settlement than the standard biological inducer (diatoms).	Heasman et al. (2004)
<i>Haliotis diversicolor</i>	Veliger (36 hrs post-fertilization)	Chemical cues (GABA, KCl)	Laboratory	Settlement was higher in KCl, with no difference between 5 mM and 10 mM (50%), while 10^{-6} M GABA had 40%. Metamorphosis was highest in 10^{-6} M GABA, and there was no difference between 5 mM and 10 mM KCl.	Li et al. (2006)
<i>Haliotis asinina</i>	Veliger (6 hours post hatch)	Chemical cues (GABA, KCl)	Laboratory	Settlement and survival were highest in 0.50 μ M GABA with 45% and 49%, respectively. Metamorphosis was not different between GABA treatments. The 6 mM KCl had higher settlement (29%), metamorphosed larvae (12%), and higher survival (29%) compared to other treatments.	Gapasin and Polohan (2004)

<i>Haliotis discus hannai</i>	Competent (crawling and exploring)	Chemical (Bromomethane, KCl)		The highest settlement and metamorphosis rates of larvae were observed in bromomethane concentrations of 600 ppm. The highest settlement and metamorphosis rates of larvae were observed in concentrations of 14–19 mM KCl.	Kang et al. (2004)
<i>Haliotis rubra</i> and <i>H. laevigata</i>	Competent (crawling and exploring)	Biological (diatoms, CCA)	Laboratory	Larvae <i>H. rubra</i> did not respond to films of any diatom species but settled on CCA (<i>Phymatolithon repandum</i>). While <i>H. laevigata</i> settled well in all diatoms and CCA, it had the highest settlement in diatoms (<i>Navicular ramosissima</i>).	Daume et al. (1999b)
<i>Haliotis australis</i>	Competent (crawling and exploring)	Biological (CCA-coated rock, diatoms)	Hatchery	The settlement on the CCA-coated rocks was more than double (8%) that of the diatoms (1%).	Moss (1999)
<i>Haliotis diversicolor</i>	Competent (crawling and exploring)	Chemical (KCl, GABA) Biological cues (diatoms, mucus, DCF).	Laboratory	Settlement and metamorphosis were highest in DFC (85%), followed by 10^{-5} M GABA and 20 mM KCl, with settlement around 60% for both concentrations, while metamorphosis was around 85%, 15% and 10 %, respectively. Survival was lowest in 10^{-2} M, followed by 10^{-4} M in GABA concentration and 40 mM and 30 mM KCl concentration.	Bryan and Qian (1998)
<i>Haliotis iris</i>	Competent (crawling and exploring)	Chemical (GABA) Biological (diatoms)	Hatchery	12 h in GABA and diatom treatment resulted in more than 60% settlement, while diatom-only treatment, took more than 40 hours to reach 50% settlement.	Moss and Tong (1992a)
<i>Haliotis rufescens</i>	Competent (crawling and exploring)	Biological (mucus, diatom) Chemical (GABA)	Hatchery	Larval settlement success differed significantly with substrate type, with mucus yielding the highest levels of successful larval settlement. High larval settlement rates and survival of juvenile.	Slattery (1992)

Abbreviations: d-aminovaleric acid (5-AVA); L-glutamic acid (GA); monosodium glutamate (MSG); Gamma-aminobutyric acid (GABA); crustose coralline algae (CCA); mucus, and diatoms and mucus (DCF).

1.6 Controlling physical factor of settlement

1.6.1 Water movement

Abalone larvae behave like passive particles in the water column or near the bottom, with uncertain control over their ability to settle on the surface or return to the water column (Boxshall, 2000). Water flow has a significant impact on their dispersal and can negatively affect the settlement of abalone larvae (Boxshall, 2000; Chick, 2010a). When the free-stream velocity is too high on the substrate surface, larvae can be passively swept back into the water column, potentially landing on unsuitable substrates (Boxshall 2000). Larval *H. rufescens* respond more favorably to chemical cues from the substrate, resulting in higher settlement success when free-stream velocity is low (Boxshall, 2000). A similar response was observed by Chick (2010a) in *H. rubra*, where larval settlement was more successful in low water flow conditions, regardless of whether a settlement cue was present.

Additionally, water flow can adversely affect the release of abalone larvae into their natural habitat. The time it takes for abalone larvae to settle and undergo metamorphosis can influence their dispersal (Chick, 2010a). If the released larvae do not settle immediately upon release, strong waves may wash them back into the water column, transporting them away from suitable habitats for settlement.

In abalone hatcheries, the technique of turning off the water flow during the settlement period to reduce velocity and increase settlement and metamorphosis has been adapted and is practised worldwide (Leighton 2008). When *H. midae* larvae are ready to settle, they are moved from plankton into settlement tanks. The water flow is turned off and only turned back on when the hatchery personnel cannot see any larvae swimming in the water column, approximately four days later (Stephen Ashlin, Wild Coast Abalone hatchery manager, pers. comm). Many larvae settle in the absence of water flow across the settlement substrata (Chick 2010a).

1.6.2 Temperature

Abalone are poikilothermic organisms, meaning that their biological processes are primarily determined by the temperature of their surrounding environment (Lopez et al. 1998, Searle et al. 2006, Kheder et al. 2010, Pedroso 2017). Temperature is the most crucial environmental factor because it regulates the main biochemical processes of metabolism and developmental rates (Samsing et al. 2016). Research indicates that abalone growth increases significantly with rising temperature (Lopez et al. 1998). However, exposure to temperatures beyond their

physiological tolerance limits can lead to stress, fatality, or slowed growth (Muller 2003; Pedroso 2017). Additionally, warmer temperatures can affect the reproductive hormones in adult abalone (Grubert & Ritar 2005).

In aquaculture hatcheries, temperature can be easily adjusted during spawning and larval development and maintained within the optimal limits for each species. In contrast, the distribution of juvenile and adult abalone in the wild corresponds to their thermal tolerance range (Leighton 1972). In temperate regions, abalone spawning typically coincides with warmer seasons that fall within the optimal temperature levels for each species. The survival of larvae in the ocean is likely dependent upon their ability to remain within a water mass of appropriate temperature and to settle in areas where temperature changes are not extreme (Leighton 1974). Variations in temperature outside a narrow range can lead to recruitment failures in abalone populations (Leighton 1974; Lopez et al. 1998).

1.7 Rational for the study

In South Africa, abalone ranching and stock enhancement of *H. midae* has been viewed as a potential fisheries tool to rehabilitate stock in depleted areas. The economic value of *H. midae* has motivated abalone farmers to invest in resources to seed abalone, to promoting resource sustainability. The South African abalone ranching, and stock enhancement guidelines (South African Government Gazette, 2010) provide investors with an exclusive right to seed and harvest the resource in a defined area, thereby maximizing their economic benefits.

Abalone larval seeding has been identified as a potential new technique for abalone ranching in South Africa. This technique would reduce costs and potentially increase reseeding efficiency. The settlement and metamorphosis of abalone larvae are essential for the success of commercial abalone ranching. However, settlement and metamorphosis of abalone larvae are inconsistent in abalone hatcheries (S. Ashlin, pers. comm, WCA) and research on improving larval settlement has, for the most part, been conducted under controlled, typically non-flow-through hatchery conditions. Biological cues, such as settlement plates coated with natural diatoms or the use of chemical cues like GABA or KCl are some of the techniques adopted in abalone hatcheries to improve settlement. There is limited information on whether this technique can be applied and could be viable for ocean seeding. There is also no information on the seeding of *H. midae* larvae.

The challenge is first to understand and then develop the necessary techniques that allow for economically viable release of *H. midae* larvae into the wild. This thesis seeks to better

determine the settlement characteristics of *H. midae* larvae to enhance the settlement of seeded *H. midae* larvae in the wild. Chemical, biological and environmental factors that influence the settlement of abalone larvae are investigated for their potential in improving the settlement of *H. midae* in the ocean, potentially laying a platform for commercial ranching of *H. midae* using seeded larvae. Aspects of this study could also be used in land-based farming hatcheries to enhance the larval settlement.

1.8 Research objective

Objective 1: To determine the effect of temperature on the rate of development and settlement of *H. midae* larvae (Chapter 3). A degree-day model can be used to predict developmental stages for *H. midae* larvae at different temperatures. Understanding when abalone larvae are competent to settle would assist in narrowing the settlement window, thereby reducing larval dispersal when seeded.

Objective 2. To investigate the effect of chemical and biological settlement cues on *H. midae* larval settlement in a flow-through system (Chapter 4). Studies on the effect of chemical and biological cues have generally focused on improving settlement and metamorphosis within controlled stagnant hatchery environments. This chapter seeks to determine the viability of using chemical cues to improve the settlement and metamorphosis of competent farmed larvae for release into the ocean by testing them in flow-through systems.

Objective 3. To determine the optimal dose of staining fluorescent dye for shell marking and its effect on larval survival and settlement (Chapter 5). The unambiguous identification of seeded abalone is essential for mark-recapture studies. Various fluorescent dyes were used to tag abalone larvae and assess larval survival and settlement at different fluorochrome concentrations and immersion periods. Chapter 5 seeks to determine a suitable fluorescent dye concentration that will mark the shell but will not negatively affect the settlement and survival of *H. midae* larvae.

Objective 4. To assess settlement success and the short-term survival of seeding abalone larvae *H. midae* in the ocean (Chapter 6). Abalone larval settlement and survival are crucial to the success of abalone larval ranching in South Africa. This chapter seeks to understand whether seeding abalone larvae along the South African coastline the potential to restock the depleted wild abalone stock and whether it could provide an option for commercial ranching operations.

Parts of the next chapter have been published as: Mzozo et al. (2021). Effect of temperature on the development and settlement of the abalone larvae *Haliotis midae*: considerations for abalone hatchery management and stock enhancement. *Journal of Shellfish Research*, 40 (1): 119-125. <https://doi.org/10.2983/035.040.0111> (Appendix 2)

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CHAPTER 2

Effect of temperature on the development and settlement of the abalone larvae *Haliotis midae*: considerations for abalone hatchery management and stock enhancement

2.1 Introduction

Abalones are poikilotherms and have a limited thermal range, with their growth and other biological processes predominately determined by the temperature of their surrounding environment (Lopez et al. 1998, Searle et al. 2006, Kheder et al. 2010, Pedroso 2017). An increase in temperature within the limits of a lower development threshold and an upper lethal temperature result in higher enzymatic reaction rates, which support a faster growth rate (Muller 2003, Pedroso 2017). Temperature, therefore, influences abalone biological rates such as spawning, developmental rates, and life span (Kheder et al. 2010). Exposure to temperatures beyond physiological tolerance limits can be stressful, fatal, or slow down growth (Muller 2003, Pedroso 2017). The larval period of *Haliotis midae* typically takes between 4 and 10 days, depending on the water temperature (Visser-Roux 2011).

In South Africa, the large-scale illegal harvesting of the wild abalones *H. midae* has caused a rapid decline in wild abalone stock (De Waal 2002), with stocks considered to be heavily depleted (DAFF 2017). To rebuild the abalone stock fishery, stock enhancement has been recommended as a fishery management tool to rehabilitate wild stocks using cultured juveniles (Godfrey, 2003). Information on stock enhancement using hatchery-raised *H. midae* juvenile abalones has been reported in several articles (De Waal & Cook 2001, Godfrey 2003) but has not been investigated using *H. midae* larvae.

Although temperature can be easily manipulated in the hatchery when rearing abalone larvae, the temperature of the natural environment may vary daily and seasonally (Muller 2003). Abalones *H. midae* occurs from St. Helena Bay on the West Coast of South Africa to the north of Port St. Johns on the East Coast (Visser-Roux 2011), where the coastline temperatures range

from an average of 13°C–25°C (Smit et al. 2013). Therefore, understanding the effect of temperature on the development and settlement of larvae is a critical first step in developing a restoration effort to seed farm-spawned larvae into the ocean.

This study was designed to test the effect of five different temperatures (14°C–22°C) on the development and settlement of abalone larvae (*H. midae*) that were reared at the hatchery. The temperature treatments used in this study are within the natural range for *H. midae*. To compare the effect of temperature on development, the data were analyzed in relation to degree days (DD). The information can be used to model larval development, thereby estimating larval competence to settle across a range of temperatures. The findings can also be used to plan production effectively in *H. midae* hatcheries.

2.2 Materials and methods

Five temperature treatments ($14 \pm 0.11^\circ\text{C}$, $16 \pm 0.4^\circ\text{C}$, $18 \pm 0.11^\circ\text{C}$, $20 \pm 0.23^\circ\text{C}$, and $22 \pm 0.02^\circ\text{C}$) with four replicates each was used to determine the effect of temperature on the developmental rate of *H. midae* larvae until settlement. The experiment was conducted at Wild Coast Abalone (Pty) Ltd farm (32° 45' 05" S 28° 16' 27" E), South Africa. Twenty-five-litre aquariums (filled to 23 L) were connected to a flow-through system, with the water exiting via a banjo sieve covered with 80 µm mesh. A banjo sieve is a filter design that allows the water to exit the larval-rearing tank via a mesh while blocking the larvae from the tank (Leighton, 2008). Each aquarium receives independent aeration and filtered (1 µm) UV-treated seawater. The photoperiod was set at 12L:12D. Ambient temperature was controlled with air conditioners and maintained at 16°C. A water chiller (Titan 2000, Aqua Medic) was used to maintain a suitable temperature in the aquariums. Throughout the experiments, the temperature in each aquarium was monitored daily (using a 0.1°C gradient thermometer).

As part of the farm's production process, fertilized abalone (*H. midae*) eggs were collected on a hatching tray and hatched in 1 µm filtered seawater maintained at 17°C. Newly hatched larvae (veliger stage) are free-swimming and swim over into the larval rearing tank (LRT), each of which received independent aeration and filtered (1 µm) UV-treated seawater. The photoperiod was set at 12:12 hours. Ambient temperature was controlled with air conditioners and maintained at 18 °C. Newly hatched larvae were transferred (approximately 50,000, 2.17 larvae/mL) into each aquarium. Seawater from the sump was coming in at 16°C, and thermal glass heaters (Xilong Aquarium glass heater, AT –700–200 W) were used to increase the temperature in individual aquariums to 18°C, 20°C, and 22°C. A water chiller (Titan 2000,

Aqua Medic) was used to reduce the temperature to 14°C. For all treatments, the temperature was initially maintained at 16°C and then gradually increased or decreased over 8 h until the desired temperature in the aquarium was reached. This allowed the larvae to acclimate to each experimental temperature.

Larval development was determined by taking triplicate 10 mL water samples from each LRT and identifying the larval developmental stage under a dissecting microscope (NSZ-606, Best Scope). Distinctive external and internal larval features were identified following descriptions by Genade et al. (1988) (Table 2.1). A developmental stage was considered to be attained when more than 50% of the larvae observed in a sample had the features associated with that stage. Larvae were allowed to develop until they reached a stage where they could settle.

Table 2.1 Developmental stages of abalone larvae *Haliotis midae* (Genade et al. 1988)

No.	Life cycle stage	Developmental stage	Description
1	Egg	Fertilization	Fertilization and discharge of the two polar bodies
2	Trochophore	Hatching	Initial larval shell secretion
3	Veliger	Pre-eye spot	Appearance of the pre-eye spot
4	Veliger	Retracted velum	Development of a short spine on the cephalic tentacle
5	Veliger	Crawling and settling	Development of the fourth tubule on the cephalic tentacle

Samples of larval development were taken from each aquarium twice a day (approximately 8 AM and 3 PM). After observing the under the microscope, the developmental stage and the time of this event from the start of the experiment were recorded. Larvae were raised until settlement, which was defined as the attachment to a benthic substrate. The settlement was considered complete once no more free-swimming larvae were detected in the aquarium.

Before the experiment, plastic sheets (15 × 15 cm = 450 cm² total surface area) were placed in outside tanks supplied with incoming seawater and allowed to develop a film of natural diatoms. On day four of the experiment, two sheets were suspended in each aquarium to help induce larval settlement. Once the larvae had settled, at around 8 AM settlement sheets were briefly removed from each aquarium and photographed (Canon camera EOS 2000D) at high resolution (≈24.1 megapixels). The number of larvae settled was counted on both sides of each sheet using DotDotGoose software (version 1.1.1, Ersts 2019). In addition to settling on the

plastic sheets, larvae also settled on the sides of the aquarium, but these were not counted. Instead, the total number was extrapolated based on the total surface area of the aquarium. The experiment was conducted for 10 days.

Temperature data loggers (UA-001–08, Onset HOBO data loggers) were placed into two aquariums of each treatment to record the temperature at 10-minute intervals throughout the study period. The data collected from the loggers were used to calculate cumulative DD (°C.day) as follows:

$$DD = \text{Time (days)} \times \text{Average (temperature } ^\circ\text{C)}.$$

Time was measured in 24 h days (n), whereas the average temperature was the average rearing temperature across n days.

Statistical analysis

Observation of developmental stages

Although the experimental design had four replicates, these replicates are irrelevant in this analysis because the stages were observed in all replicates simultaneously (no variation among replicates). To study the development rate of larvae in relation to temperature, a generalized linear model (R Core Team 2021) was used to examine how temperature determines the hour or DD at which each development stage is observed. With hour or DD as the response variable, separate slope coefficients (i.e., development rates) were obtained for each developmental stage by modelling the interaction effect of temperature with a factor variable representing the developmental stage. Although temperature treatment is essentially a factor with five levels in the experimental design, temperature was used as a continuous predictor variable in this model because there were too few data for a model with two interacting factor variables. A Gamma distribution family was used because both hours and DD are continuous variables that can only be positive. The overall significance of the predictors was expressed as an analysis of variance (ANOVA, R function "Anova" in the "car" package, Fox & Weisberg, 2019).

Temporal settlement of larvae at each temperature exploratory

Exploratory analyses show that the number of larvae settling over time appears to be nonlinear (Fig. 2.3); however, preliminary analyses with nonlinear models (R function "nls," R Core Team, 2021) failed (e.g., the Lomo lino function) or fitted the data very poorly (e.g., a logistic function), as the number of observations over time (maximum seven for 20°C and 22°C) was

too few to reasonably fit a nonlinear function. Furthermore, the abundance of settled larvae observed at the conclusion of the experiment differs substantially among temperature treatments (Fig. 2.2).

Therefore, to study larval settlement over time at different temperatures, a linear random-effects model (R package "lme4," Bates et al. 2015) with the Poisson distribution family was used to relate the number of larvae counted on the sheets to two random effects—hours (random slope) and temperature as a factor (random intercept). This model supplies a unique slope (settlement rate) and intercept (abundance reached) for each temperature treatment. Even though this random-effects model is fairly complex, preliminary analyses showed that it fitted the response variable better (the lowest Akaike's information criterion value) than other models where either or both hours and temperature were modelled as fixed effects. An analysis of deviance was used to obtain a Likelihood ratio chi-square value (hereafter χ^2) and approximate P value for hours and temperature by comparing models with or without the relevant random effect.

2.3 Results

The rate of larval development varied with temperature, suggesting that temperature plays a significant role in the development of *H. midae*. With each 2°C change in temperature, there was an increase in the rate of development. Temperature is a strong predictor of when a particular stage of development is reached, both when considering the time (hours) until a stage is reached ($\chi^2 = 215.34$, $p < 0.0001$) and DD ($\chi^2 = 112.59$, $p < 0.0001$). Specifically, each developmental stage is observed significantly earlier, or at a lesser DD value, as temperature increases (Table 2.1, Fig. 2.2).

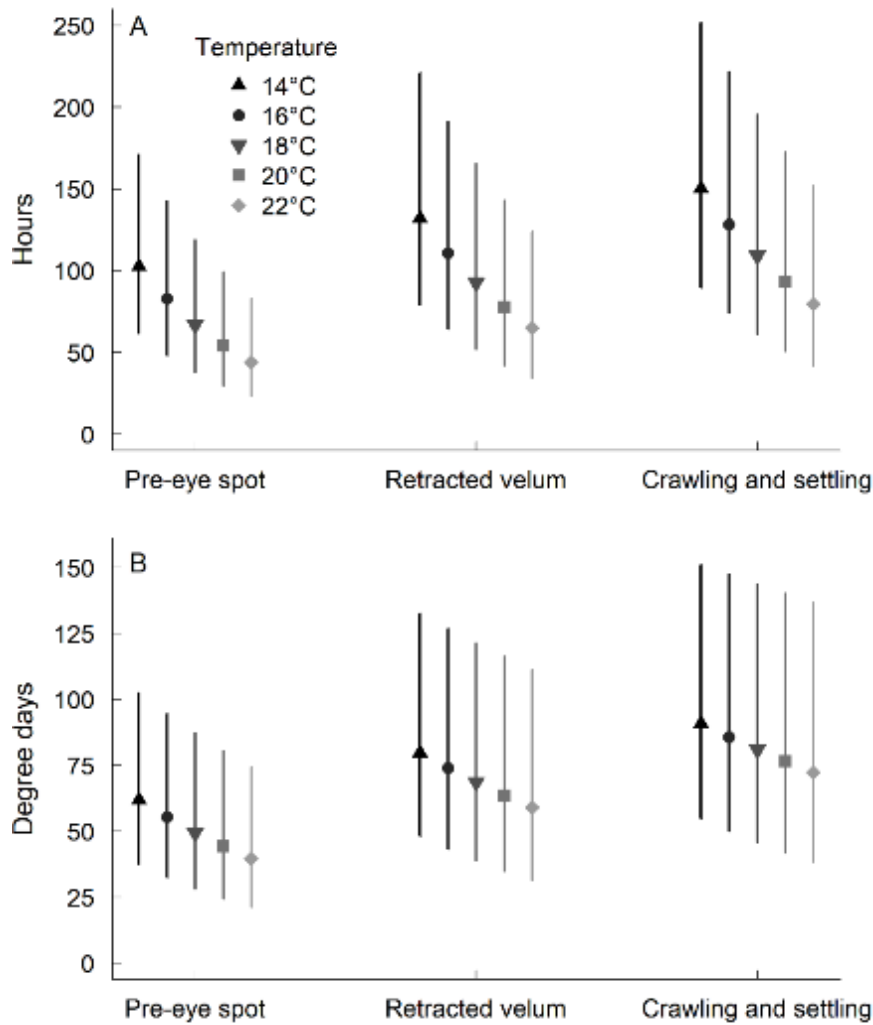
Table 2.1 Intercept with the slope coefficients* of the relationship between hours/DD and temperature at each developmental stage for *Haliotis midae* larvae.

	Hours	DD
Intercept	456.530 (342.916; 608.980)†	135.167 (101.932; 179.573)
Pre-eye spot	0.899 (0.885; 0.913)	0.946 (0.931; 0.961)
Retracted velum	0.915 (0.900; 0.930)	0.963 (0.948; 0.978)
Crawling and settling	0.924 (0.909; 0.939)	0.972 (0.956; 0.988)

DD, degree-day.

* Coefficients were back-transformed from the model's log scale.

† 95% confident interval in the brackets.



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Figure 2.2 The predicted hour or degree-days at which each developmental stage of *Haliotis midae* larvae was observed at each temperature, based on generalized linear models. The error bars are 95% confidence intervals.

The results of developmental time showed that *H. midae* larvae developed until they were competent to settle at a wide range of temperatures (14°C–22°C). The model of larval settlement at different temperatures is significantly influenced by both the random intercept term denoting overall settled larval abundance differences between temperatures ($\chi^2 = 33\,788.4$, $p < 0.0001$) and the random slope term denoting different rates of settlement at different temperatures ($\chi^2 = 8\,634.2$, $p < 0.0001$). The overall abundance of settled larvae differed substantially between treatments (Table 2.2, Fig. 2.3). For example, the maximum number of settled larvae observed at 22°C (on average about 52% of the total introduced at the beginning of the experiment) is more than twice the maximum number of settled larvae observed at 20°C (average 22% of the total). Larval settlement increased over time for all temperatures (back-

transformed slope coefficient > 1), except at 14°C where it decreased over time (<1) (Table 2.2, Fig. 2.3). Furthermore, for temperatures between 16°C and 22°C , the rate of the settlement was not consistently faster for warmer temperatures (the slope is not steeper with an increase in temperature (Table 2.2). However, these slope coefficients are likely not helpful in describing the effect of temperature because an increase in the number of settled larvae mainly happens at early stages before levelling out (Fig. 2.3), and the linear model cannot account for this nonlinear pattern. Therefore, the current model and data cannot be used to predict larval settlement in general. To create a predictive model, more observations over time are needed to allow a nonlinear model to be fitted.

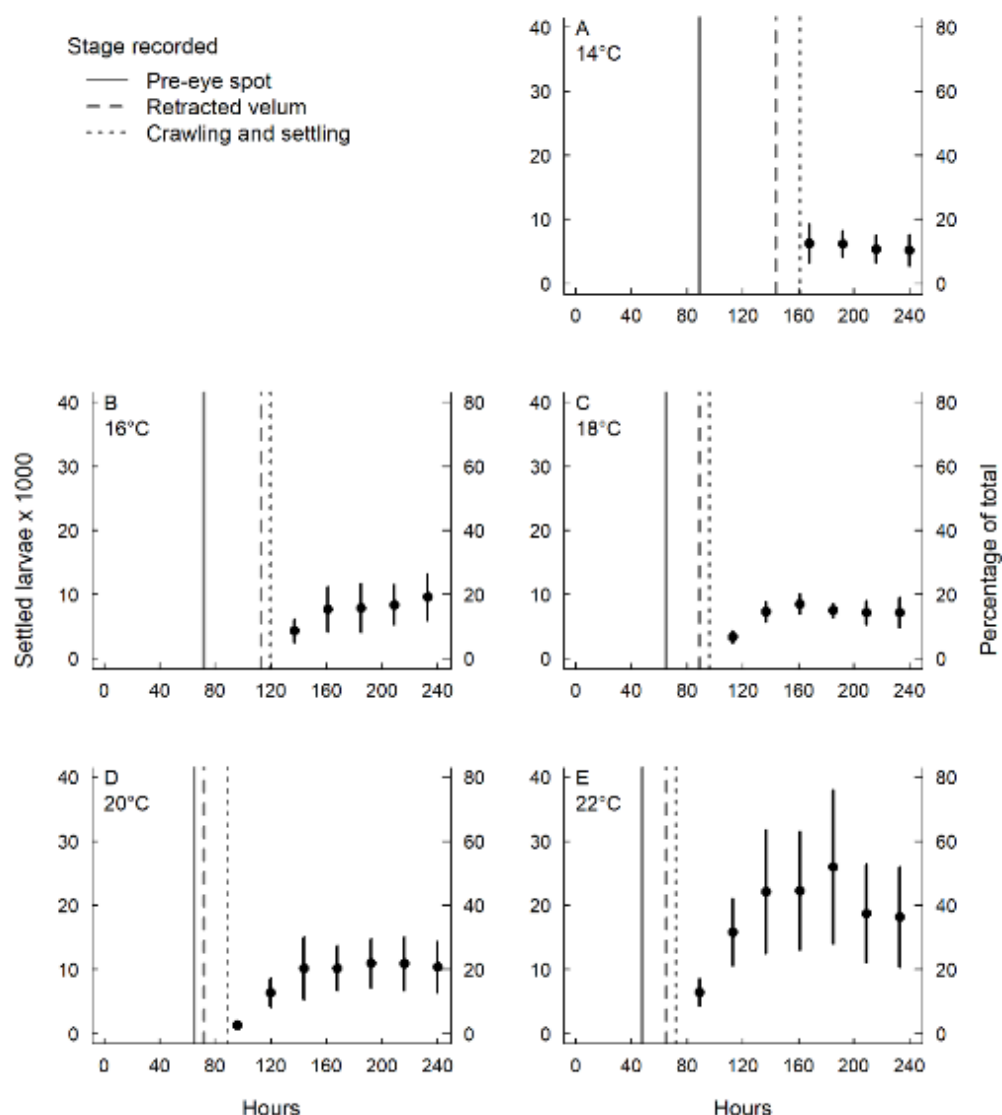


Figure 2.3 The estimated mean number (%SD) of larvae settled. Equivalent percentage, out of ca 50,000 larvae initially added to each aquarium, is indicated on the right-hand y-axis. The vertical line depict the observed hour at which greater than 50% of the larvae had reach each particular stage

Table 2.2 Intercept* and slope coefficients* for the number of larvae (*Haliotis midae*) settled over time (hours) at each temperature

Temperature (°C)	Intercept	Slope
14	1.107 (1.083; 1.131)†	0.881 (0.859; 0.904)
16	1.243 (1.226; 1.260)	1.318 (1.297; 1.339)
18	1.216 (1.202; 1.230)	1.148 (1.133; 1.162)
20	1.533 (1.518; 1.547)	1.337 (1.325; 1.350)
22	3.428 (3.406; 3.450)	1.175 (1.168; 1.182)

* Coefficients were back-transformed from the model's log scale.

† The lower and upper 95% confidence intervals in brackets.

By the end of the experiment, no larvae were swimming in the aquariums at temperatures 18°C, 20°C, and 22°C; however, at 14°C and 16°C, there were still 0.2 ± 0.06 and 0.15 ± 0.04 larvae.mL⁻¹, respectively. Abalone larvae which had settled on the sheets in the 18°C, 20°C, and 22°C treatments had metamorphosed, whereas larvae in the 14°C and 16°C treatments had not.

2.4 Discussion

When food is not a limiting factor, temperature significantly impacts the development rate of many organisms (Scharf et al. 2015). It controls the principal biochemical processes of metabolism and regulates developmental rates (Samsing et al. 2016). Because temperature controls abalone growth, it is considered an ecological resource and can be quantified using a DD approach (Chezik et al. 2014). The DD approach works best when describing the development in endogenous feeding larvae because the development is only controlled by age, temperature, and yolk quality (Malzahn et al. 2003). The DD approach assumes that there is a linear relationship between temperature and growth (Malzahn et al. 2003, Chezik et al. 2014). It allows for developmental comparison between temperatures by reducing the effect of age (days), meaning that larvae with the same age but not temperature history can be directly compared (Malzahn et al. 2003). In this study, the development of *H. midae* larvae at different temperatures was compared using the DD approach. The data presented in this article show a significant influence of temperature on the development of *H. midae* larvae. Abalone larvae at the same age (days) had a faster developmental rate at a higher temperature (22°C) than at a lower temperature (14°C). Chezik et al. (2014) reported that the DD approach could be used to predict the growth of fish larvae as it outperforms calendar time—it directly measures the thermal opportunity. It predicts growth with accuracy within two days. Therefore, the DD approach can be used for long-term planning in abalone farm hatcheries.

Temperature controls the principal biochemical processes of metabolism and regulates developmental rates (Samsing et al. 2016). Using the DD approach to normalizing the data clearly showed the temperature dependence of the development of *H. midae* larvae. In the present study, the developmental period for *H. midae* larvae to reach the various stages of development decreased with an increase in temperature across the range tested. The rate of larval development increased with temperature over the range of 14°C–22°C, with every 2°C change in the temperature significantly increasing the rate of development. Larvae cultured at 22°C completed development and were competent to settle in 3.7 days (80 DD), whereas those held at 14°C had just developed a pre-eye spot (Fig. 2.2). Similar results were observed by Muller (2003), Grubert & Ritar (2005), Onitsuka et al. (2010), Visser-Roux (2011), McCormick et al. (2016), Pedroso (2017) who reported that the developmental rate was most rapid at higher temperatures and slowest at lower temperatures. This study showed that *H. midae* raised at 20°C completed development and was competent to settle in 4 days (77.88 DD). These findings contradict the results of (Genade et al. 1988), who found that *H. midae* larvae raised at 20°C were competent to settle in 5 days. This could be attributed to possible genetic differences between the broodstock. The abalone broodstock used in this study were F1 individuals from Wild Coast Abalone farm's hatchery, whereas Genade et al. (1988) used adult abalone collected from the ocean. On the farm, hatchery managers select the fastest-growing abalones to use as F1 broodstock (S. Ashlin, WCA farm hatchery manager, 2019, personal communication). This difference in the larval development rate could also be attributed to possible nutritional differences in the broodstock of the two studies and possibly owing to the different larval culture techniques used. Larval rearing techniques have improved considerably since Genade et al. (1988) conducted their experiment, which might have led to faster growth in the present study.

No specific attention was given to the number of mortalities occurring during the planktonic stages of *H. midae* development. We observed that patches of dead larvae (referred to in abalone hatcheries as "spotting") occurred more frequently between the developmental stage of pre-eye spot development and just before they were competent to settle. Genade et al. (1988) reported that no reliable data were obtained on larval (*H. midae*) mortality during the experiments—larvae reared at 17.5°C and 20°C developed until they were competent to settle. In this experiment, larvae also developed until they were competent to settle in all treatments.

Once larvae are competent to settle, they must move from being planktonic to benthic larvae and thus begin searching for a suitable substrate to settle and metamorphose (Genade et al.

1988, Freeman 2001). The current study found a positive response of larval settlement in all temperature treatments except for the 14°C treatment. Larvae raised at 22°C had the highest percentage of initial settlement, whereas those at 14°C had the lowest (see Fig. 2.3). Similar results were reported by Nosrolahi (2012) on the bay barnacle *Amphibalanus improvises*, showing that the highest settlement occurred at 20°C with almost zero at 12°C. This was within the natural temperature range of *A. improvises* between 0°C in winter up to 23°C during late summer (Nosrolahi 2012). Leighton (1972) reported a higher percentage of settled *Haliotis sorenseni* larvae at warmer temperatures between 12°C and 20°C and zero settled larvae at 10°C. At the same time, larvae remained alive in all treatments after 15 days. The results by Leighton (1972) indicate that poor settlement of larvae does not mean mortality, which is the case in the present study with a poor settlement at 14°C. These results indicate that apart from chemical and physical inducers for settlement, temperature also plays a significant role in settlement of *H. midae* larvae. Warmer temperatures result in a better settlement within the optimal temperature range for the species.

After settlement, when conditions are unsuitable for metamorphosis, competent larvae attach to the substrate, crawl, and swim to the surface (Morse 1991, Moss & Tong 1992b, Moss 1999). The current study's results show that at 14 and 16°C, larvae were still swimming at the end of the experiment after ten days, with more swimming larvae at 14°C than at 16°C. Moss (1999) reported that cultured *Haliotis australis* larvae appear to settle but then swim off the surface when seawater temperature was between 10°C and 12°C, and this behaviour lasted up to 8 days. Roberts and Lapworth (2001) reported that *Haliotis iris* larvae were competent to settle seven days after fertilization but could still survive for 34 days without metamorphosis; however, only 4% of post-larvae survived after completing metamorphosis 34 days later. As this experiment was terminated after ten days, larvae at 14°C and 16°C may still have had a chance to metamorphosis sometime later. However, the effects of delayed metamorphosis on *H. midae* larvae need to be quantified to determine the dispersal window in the ocean when seeding larvae at lower temperatures.

When conditions are suitable, larvae cast off their swimming velum and change from being swimming larvae into benthic juveniles undergoing metamorphosis. Metamorphosis is an irreversible process and involves the mantle membrane separating from the larval shell and moving to the shell edge, after which juvenile shell growth commences (Leighton 2008). Larvae reared at 18°C, 20°C, and 22°C had metamorphosed, and no larvae were swimming in the water column at the end of the experiment. Meanwhile, those at 14°C and 16°C had not yet

metamorphosed, and larvae were still swimming in the water column at approximately 10% of the initial density. Nosrolahi (2012) reported that when the barnacle *A. improvisus* larvae were cultured at higher temperatures, it increased the metabolic rate to support a high growth rate. By contrast, more energy costs are associated with calcification (shell formation) in cold water for those maintained at low temperatures. Therefore, they allocate more energy toward vital processes such as somatic mass maintenance at the expense of calcification. This might be the case in this study; abalone larvae at 14°C and 16°C appeared competent to settle but may have been portioning more energy toward maintaining their somatic mass instead of calcifying their shell.

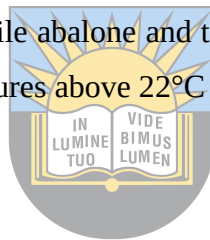
Natural diatom films are commonly used to induce larval settlement in abalone hatcheries worldwide (Roberts 2001). Biofilms developed from the natural diatoms in the incoming seawater were used in this study, and therefore, the species composition was not controlled and recorded. The percentage of settled larvae ranged between $2.56\% \pm 0.61\%$ and $51.98\% \pm 23.95\%$, which is substantially higher than what is considered acceptable settlement ($\approx 5\%$) by commercial hatcheries (S. Ashlin, WCA farm hatchery manager, 2019, pers comm); however, juveniles are only counted in the hatchery three months after settlement. A study by Daume et al. (1999b) reported that the percentage settlement of *Haliotis laevigata* larvae on different species of cultured diatoms ranged between 15% and 45% after 15 days. Similarly, Li et al. (2006) reported that the larval settlement of *Haliotis diversicolor* was 25% on plastic sheets colonized by natural diatoms after six days. This suggests that although settlement can be highly variable, the percentage of settlement of *H. midae* in this study was within the range of other abalone species.

The results from this study on modelling DD of larval development could also be used in abalone farm hatcheries. The DD model can be used to predict developmental stages for *H. midae* larvae at different temperatures using data presented in this study. This information is crucial for effectively controlling production in the hatchery as it impacts the spawning schedule, diatom availability, nursery space, and logistics. The results are also relevant to abalone larval stock enhancement; seeding *H. midae* larvae at warmer temperatures will result in a higher settlement, reducing the dispersal of larvae outside the seeding area. Rearing *H. midae* larvae at 18°C and 22°C might also result in faster juvenile growth. This is because an increased temperature within an optimal range of the species may increase the metabolic rate at a level that can still support an increase in growth (Pedroso, 2017). This hypothesis is supported by Britz et al. (1997), who reported that the growth rate of juvenile *H. midae*

increased across a 12°C–20°C temperature range. This will be advantageous for hatcheries and stock enhancement programmes because this temperature range (18°C and 22°C) corresponds to the sea temperature range experienced by *H. midae* within its natural environment.

In conclusion, temperature controls the planktonic development, settlement, and metamorphosis of *H. midae* larvae. Moreover, rearing *H. midae* larvae at 22°C increased the developmental rate and yielded a high settlement percentage. This result will benefit the abalone stock enhancement programmes—seeding larvae at warmer temperatures will increase the growth rate of abalone larvae and juveniles. It will reduce the potential of dispersing outside the seeding area and, therefore, increase the potential success of abalone ranching and stock enhancement programmes. Last, the warmer temperature will increase the percentage of initial settlement leading to metamorphosis of *H. midae* larvae, thereby increasing the possibility of rebuilding the wild stock.

Further research is necessary to investigate the long-term effect of temperature on the development of recently settled juvenile abalone and to assess the physiological effects of *H. midae* larval development at temperatures above 22°C and below 14°C.



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Parts of the next chapter have been published as: Mzozo et al. (2023). The use of chemical and biological settlement cues in enhancing the larval settlement of abalone (Haliotis midae): Implications for hatcheries and ocean ranching. Journal of the World Aquaculture Society, 1–16. <https://doi.org/10.1111/jwas.13001>. (Appendix 3)

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CHAPTER 3

The use of chemical and biological settlement cues in enhancing the larval settlement of abalone (*Haliotis midae*): implications for hatcheries and ocean ranching

3.1 Introduction

The South African abalone (*Haliotis midae*) is the most commercially valuable species in the local aquaculture industry DAFF (2017). Its high value has driven illegal fishing, resulting in the decline of the wild stock population, which has been well documented (De Waal 2002, Godfrey 2003, Raemaekers et al. 2011, Witte 2017). Both stock enhancement and land-based abalone farming require a large number of hatchery-produced larvae (Li et al. 2006). Stock enhancement programmes typically use land-based farmed juvenile abalone (De Waal 2002, Godfrey 2003); however, the production cost of raising juvenile abalone on a land-based abalone farm is high; therefore, the seeding of abalone larvae has been considered a viable alternative. One of the significant constraints with seed production on abalone farms is poor larval settlement and metamorphosis, which results in low post-larval survival (Gapasin & Polohan 2004). Li et al. (2006) reported that a maximum of 10% of larvae develop into metamorphosis while the remaining larvae die. Therefore, reducing the time between larval release and settlement in the wild should reduce dispersion outside the seeding area and increase the chances of settlement.

Many studies have focused on improving settlement and metamorphosis in commercially valuable abalone species from chemical cues such as γ -aminobutyric acid (GABA) (Gapasin and Polohan, 2004; Li et al., 2006; Moss and Tong, 1992a; Stewart et al., 2008), potassium ions (Yool et al. 1986, Gapasin & Polohan 2004, Li et al. 2006, Yu et al. 2010), bromomethane (Kang et al. 2004), and biological cues such as natural biofilms (Gordon et al. 2004) and conspecific mucus (Slattery 1987).

Potassium chloride (KCl) is one of the most commonly used chemical cues and is an effective chemical settlement inducer that is also inexpensive and readily available. Literature

investigating the effect of KCl has generally focused on improving settlement and metamorphosis within a controlled hatchery-like environment. For example, Bryan and Qian (1998) exposed competent *Haliotis diversicolor* larvae to a chemical cue for 96 h without water exchange. Likewise, Gapasin and Polohan (2004) exposed *Haliotis asinina* early veliger larvae (5–6 h post-hatch) to KCl until post larvae, 72 h post initial exposure, without any water exchange. Li et al. (2006) exposed *H. diversicolor* trochophore larvae to KCl until settlement and metamorphosis. In abalone hatcheries, water flow is turned off during settlement until no larvae are observed swimming in the water column, usually after ± 72 hours (S. Ashlin, WCA hatchery manager, pers. comm). While previous studies have emphasized the application to aquaculture, little is known about the viability of using chemical cues to improve the settlement and metamorphosis of competent farmed larvae released into the ocean.

This study aimed to investigate the settlement and metamorphosis of competent *H. midae* larvae exposed to KCl and the benthic diatom *Nitzschia*. This was to approximate the methodology used to enhance larval survival and settlement if larval seeding in the ocean is shown to be viable. First, an optimal concentration of KCl to induce *H. midae* larval settlement was determined. Although the inducing effect of KCl has been tested on various abalone species, the responses of individual species are highly variable, even to the same inducing cue (Li et al. 2006). The optimal chemical concentration for each species should be known to maximize the settlement and metamorphosis. Next, using the optimal concentration of KCl, the effect of acute and chronic exposure of competent *H. midae* larvae to KCl and *Nitzschia* (free-swimming) before ocean seeding on the reef was investigated. This was achieved through laboratory experiments designed to simulate ocean seeding after exposure in captivity by comparing the larval settlement during and after exposure for different exposure durations. The following hypotheses were tested: (1) chronic exposure of *H. midae* larvae to settlement cues (KCl and *Nitzschia*) before seeding on the reef will increase settlement and metamorphosis compared to acute exposure to the cues; (2) settlement and substrate cue: exposure of abalone larvae to a combination of settlement cues and suitable substrate will result in more significant settlement and metamorphosis.

3.2 Materials and methods

Abalone larval rearing

As part of the farm's production process, fertilized abalone (*H. midae*) eggs were collected on a hatching tray and hatched in 1µm filtered seawater maintained at 17°C. Newly hatched larvae (veliger stage) are free-swimming and swim over into the larval rearing tank (LRT), each of which received independent aeration and filtered (1 µm) UV-treated seawater. The photoperiod was set at 12:12 hours. Ambient temperature was controlled with air conditioners and maintained at 18 °C. Larval development was determined by taking triplicate 10 mL water samples from each LRT and identifying the larval developmental stage under a dissecting microscope (NSZ-606, Best Scope). Distinctive external and internal larval features were identified following descriptions by Genade et al. (1988) (Table 3.1). A developmental stage was considered to be attained when more than 50% of the larvae observed in a sample had the features associated with that stage. Larvae were allowed to develop until they reached a stage where they could settle.

Table 3.1 Developmental stages of abalone larvae *Haliotis midae* (Genade et al. 1988)



No.	Life cycle stage	Developmental stage	Description
1	Egg	Fertilization	Fertilization and discharge of the two polar bodies
2	Trochophore	Hatching	Initial larval shell secretion
3	Veliger	Pre-eye spot	Appearance of the pre-eye spot
4	Veliger	Retracted velum	Development of a short spine on the cephalic tentacle
5	Veliger	Crawling and settling	Development of the fourth tubule on the cephalic tentacle

Abalone larvae (*H. midae*) that are competent to settle (refer to table 2.1) were transported in 25 L buckets from Wild Coast Abalone (Pty) Ltd farm to Kingfish Enterprises (Pty) Ltd farm, East London, South Africa.

Diatom coated settlement sheet

Before the experiment, Wild Coast Abalone Farm (Pty) Ltd supplied stock cultures of *Amphora* sp., *Nitzschia* sp., and *Navicula* sp. diatoms, which were used in these experiments. The cultures were maintained at Kingfish Enterprises (Pty) Ltd in a temperature-controlled (20 ± 1

°C) laboratory using 1 µm filtered seawater. Microalgae cultures were fertilized using Varicon aqua solution, Cell-Hi F2F, and sodium metasilicate while the photoperiod was set at 24 hr light under fluorescent lighting.

Plastic sheets (15×10 cm = 300 cm² total surface area) were placed in tanks containing a mixture of equal parts of each cultured diatom (33.3% each culture) (*Amphora* sp., *Nitzschia* sp., and *Navicula* sp.) and fertilized seawater (Varicon aqua Cell-Hi F2P, sodium metasilicate). Aeration was provided, and to maintain good water quality, a 75% water exchange was performed twice a week, and more cultured diatoms were added. Diatoms were allowed to settle and proliferate for three weeks, yielding a thin diatom biofilm on the surface of the sheets.

Experimental system

The experimental systems were designed as a water bath system, with each aquarium suspended in temperature-regulated water, maintaining the system's temperature. The system was connected to the flow-through system, with the water exiting via a banjo sieve covered with 80 µm mesh. Each aquarium receives independent aeration and filtered (1 µm) UV-treated seawater. The photoperiod was set at 12L:12D. A water chiller (Titan 2000, Aqua Medic) was used to maintain a suitable temperature in the aquariums. Throughout the experiments, the temperature in each aquarium was monitored daily (using a 0.1°C gradient thermometer). Two settlement sheets coated with diatoms were suspended in an aquarium. The treatments were randomly allocated using a 10 L aquarium for experiment one and a 15 L aquarium for experiments two and three.

i. The optimal concentration of KCl for H. midae larval settlement

Bryan and Qian (1998) suggested that the optimal concentration for potassium ions to induce larval settlement for *H. diversicolor* ranged between 10–20 mM. For this study, a control (with no KCl) and three KCl (Sigma–Aldrich, South Africa) concentrations (10, 15, and 20 mM) were used. A KCl stock solution was prepared using 1 µm filtered seawater and diluted for each treatment. Clean-filtered seawater was used in the control treatment, with three replicates per treatment. Water flow was switched off in all treatments (including the control) before adding the KCl. Two settlement sheets (15 × 10 cm = 300 cm² total surface area) coated with diatoms were suspended in each aquarium to provide an adequate larval settlement in each treatment. Approximately 20 000 (2 larvae.mL⁻¹) abalone larvae were added to each 10 L aquarium, and the larvae were exposed to the KCl for six hours, after which the water flow was turned back on. During and after the exposure, once the larvae had settled, settlement sheets

were briefly removed from each aquarium and photographed (Canon camera EOS 2000D) at high resolution (≈ 24.1 megapixels). The number of larvae settled was counted on both sides of each sheet using DotDotGoose software (version 1.1.1, Ersts 2019). The experiment ran for 94 h, with counting occurring at increasing intervals. To determine the number of larvae still swimming in the water column, at the end of the experiment (94 h), a triplicate of 10 mL water samples was collected from each aquarium and counted under a dissecting microscope (Best Scope-3040T, Lasec South Africa).

ii. *The effect of the combination of settlement cues (chemical and biological) and exposure periods on larval settlement*

This experiment was performed to determine the effect of the 12- and 24-hour exposure period and the effect of different inducing cues on the larval settlement and metamorphosis. Three experimental treatments were used – larvae exposed to KCl for 12 hours, 24 hours exposure to KCl, 24 hours exposure to a combination of KCl and planktonic *Nitzschia*, and two control treatments where water flow was stopped for 12 hours and 24 hours, respectively but with no KCl or planktonic *Nitzschia* exposure. Three replicates were used for each treatment. The optimal concentration of KCl (10 mM) determined in the first experiment was used for all experimental treatments in this second experiment. Diatom cell concentration was calculated using a haemocytometer under a microscope, and a serial diluting of diatoms was done until the required number of cells was achieved. For the combination of KCl and *Nitzschia* exposed for 24 hours, 120 cells.mL⁻¹ of *Nitzschia* was added per aquarium. Approximately 30,000 larvae (± 2 mL⁻¹) were added to each 15 L aquarium. Water flow was stopped for 12 or 24 hours, exposing larvae to the treatment cues for the entire period, including the control treatment. Water flow was then restored, and the number of settled larvae was counted by taking pictures following the method described in the Experiment i- counting settled larvae.

At the end of the experiment, swimming larvae were counted as described in experiment one, and after counting, water was carefully siphoned from each aquarium to avoid disturbing those larvae that might have settled on the surfaces of the aquarium. Settled abalone larvae were then removed from the sides of the aquarium by adding 1 M MgSO₄ (Magnesium sulfate), which acts as a muscle relaxant in abalone (White et al. 1996). Abalone were removed by gently spraying them with seawater, concentrating them, and taking triplicate 10 mL samples from each aquarium, which were counted under a dissecting microscope. After 94 hours, the experimental settlement sheets were observed at x10 magnification under the dissecting

microscope (Best Scope–3040T, Lasec South Africa) to determine the number of metamorphosed larvae on each sheet. Metamorphosed larvae were identified by shell growth (Leighton 2008).

iii. Larval settlement in response to KCl and planktonic *Nitzschia* sp. after short exposure

This experiment determined the larvae's settlement response and metamorphosis after a brief exposure to the settlement cues. Five treatments were tested: (a) KCl only, (b) planktonic *Nitzschia* only, (c) a combination of KCl and planktonic *Nitzschia* (d) a control (with no KCl or *Nitzschia*), (e) a reference control with the treatment that achieved the highest settlement in experiment two (24 hours exposure to a combination of 10 mM KCl and planktonic *Nitzschia*). Again, the optimal concentration of KCl from the first experiment was used for all experimental treatments in the third experiment. All treatments were set up as described in the second experiment, with three replicates for each treatment. Approximately 30,000 larvae were added to each 10 L aquarium (± 3 larvae.mL⁻¹) and exposed to the respective treatment for 60 minutes. After 60 min, the settlement inducers were removed by gently collecting the larvae from each aquarium on a 60 μ m screen. The screen was carefully removed from the bucket, and the larvae were removed by rinsing the screen into the 15 L aquarium with the flow-through system to assess post-exposure settlement. Two settlement sheets (one sheet coated with diatoms and the other uncoated) were added post-flushing the settlement cues. The immediate settlement post-exposure was assessed after two, then at six-hour intervals for 24 hours and then at 12-hour intervals until 74 hours. The reference and experimental treatment were set up in a 15 L aquarium, and settlement sheets were counted pre- and post-exposure. Metamorphosis was assessed following the same method as described in the second experiment.

Statistical analysis

For all three experiments, generalized linear models were used to relate the number of larvae counted on the sheets to the duration of the experiment in hours as a continuous variable, the treatment as a factor variable, and the interaction effect between hours and treatment (R statistical software, (R Core Team 2021). This model gives separate intercepts and slopes for each treatment; therefore, the estimated change in settlement over time could be plotted for each treatment in an experiment. The Poisson distribution was used to model larval count data; therefore, the intercept and slope estimates were "back-transformed" to the original scale from the log scale. This means that for each treatment, the line equation was given by the power

function: $Larvae \sim Intercept(Slope^{Hours})$. Back-transformed slope estimates below 1 indicate negative relationships, and above 1 indicate positive relationships and non-significant relationships have slope estimates with 95% confidence intervals that overlap 1. Further, the model output of each experiment was expressed as an analysis of covariance (ANCOVA) table with the likelihood ratio chi-square (χ^2) test statistic, and the p -value <0.05 regarded as significant (R package 'car') (Fox & Weisberg 2019). Model diagnostic plots of residuals vs. fitted values indicate mostly random residual values except for experiment 2, where there is an increased residual variation at higher fitted values. However, for all experiments, Poisson models fit better to the data compared to other options that were checked in preliminary tests, i.e., count data with a negative binomial distribution, percentage settlement with a binomial distribution, and an arc-sine transformed percentage settlement with a Gaussian distribution.

The number of metamorphosed larvae is a proportion of the number of settled larvae. Therefore, we use a generalized linear model with a binomial distribution family to analyze the variation in metamorphoses across treatments for experiments 2 and 3. In this model, the response variable is a binomial variable that combines the number of successes (metamorphosed larvae) and the number of failures (larvae that did not metamorphose), which allows the model to take into account the total number of settled larvae in each treatment. To avoid pseudo-replication, the number of larvae counted on both sheets was pooled from each treatment replicate. An exploration of the data suggests that the chances for metamorphosis are generally very high, as nearly all larvae had metamorphosed in most replicates. Therefore, a complementary log-log link was used in the model, which is appropriate when the probability of an event is very high or very low. The results for each experiment were expressed as an analysis of variance, and a Tukey Honestly Significant Difference (HSD) post hoc test was performed to reveal pairwise differences between treatments (R function 'glht' from R package 'multcomp,' (Hothorn et al. 2008).

3.3 Results

i. ***The optimal concentration of KCl to induce H. midae larval settlement***

Larval settlement differed significantly among concentrations (different intercepts), and larval settlement also changed significantly over time at different rates (different slopes) in all concentrations (Tables 3.2 and 3.3). During the six hours that larvae were exposed to KCl, the larval settlement was highest in the 10 mM KCl treatment and lowest in the 20 mM KCl treatment (Fig. 3.1 and the intercepts in Table 3.3). However, larval settlement decreased over

time if exposed to 10 mM KCl, while it increased over time in the other treatments, with the steepest increase occurring in the control treatment (Fig. 3.1, Table 3.3). Consequently, after 16 hours (Fig. 3.1), larval settlement in control was higher (5865 ± 2302 SD, averaged over all values recorded between 23 h and 94 h) compared to all other treatments (10 mM: 3963 ± 2302 SD, 15 mM: 2010 ± 951 SD, 20 mM: 2100 ± 1069 SD). No larvae were found swimming in the water column at the end of the 94-hour experiment.

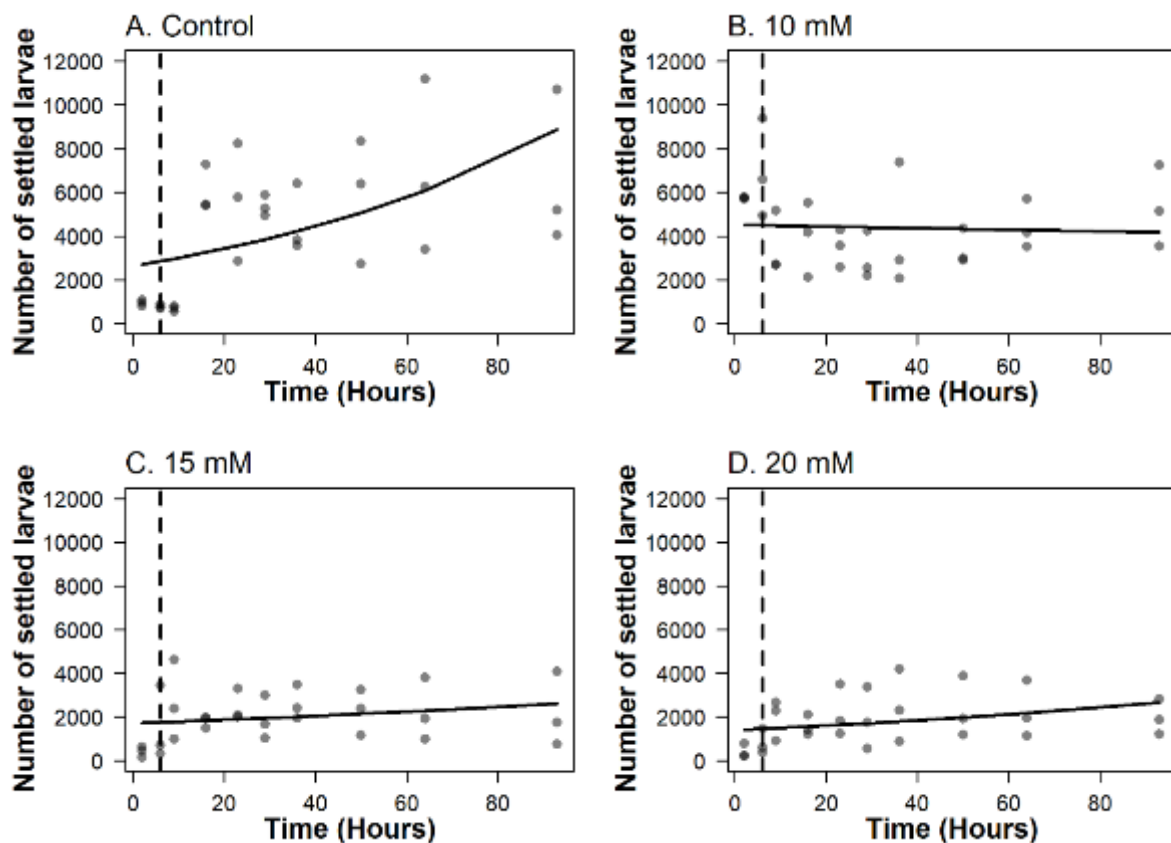


Figure 3.1 Number of settled abalone (*Haliotis midae*) larvae during and post-exposure to three different concentrations of potassium chloride (10, 15, and 20 mM).

Table 3.2 Analysis of covariance output for all three experiments. The χ^2 test statistic is indicated for each term, together with the significance indicated symbolically.

Experiment	Hours	Treatments	Interaction
1. Different concentrations	12152****	60689*****	10929*****
2. Different exposure periods	1160.5****	33040.4****	21025.4*****
3. Different cues	2565.308*****	27027.1****	187.7*****

Symbol: **** <0.0001

Table 3.3 Estimated slopes and intercepts for each KCl concentration, with 95% confidence intervals in brackets, that had been back-transformed from the log to the original scale

Concentration	Intercept	Slope*
0	2643.569 (2618.744; 2668.63)	1.0131 (1.0129; 1.0133)
10	4521.506 (4464.846; 4578.884)	0.9991 (0.9989; 0.9994)
15	1712.026 (1684.935; 1739.552)	1.0046 (1.0042; 1.0049)
20	1398.709 (1375.438; 1422.374)	1.0069 (1.0066; 1.0073)

*Slope estimates > 1 represent positive relationships, and slopes < 1 represent negative relationships.

ii. The effect of the combination of settlement cues (chemical and biological) and exposure periods on larval settlement

Larval settlement differed significantly among different exposure times, and larval settlement also changed significantly over time, with the rate of change differing among different exposure times in all concentrations (different intercepts and slopes, Tables 3.2 and 3.4). Initially, the larval settlement was highest in the treatments where larvae were exposed to a combination of 10 mM KCl and *Nitzschia* for 24 hours and where larvae were exposed to 10 mM KCl for 12 hours (see intercepts in Table 3.4). In comparison, both control treatments had significantly lower initial larval settlement (see intercepts in Table 3.4). Therefore, the combination of 10 mM KCl and *Nitzschia* exposure for 24 hours was used as the reference control in the third experiment. However, larval settlement in the combined 24-hour 10 mM KCl and *Nitzschia* treatment and the 12h 10 mM KCl treatment declined over time, while larval settlement increased over time in the 24-hour 10 mM KCl treatment and controls (Fig. 3.2 and slopes in Table 3.4). Consequently, after 24 hours (Fig. 3.2), larval settlement in the 12 hours control was highest (1490 ± 820 SD averaged over all values recorded between 27 hours and 94 hours), followed by the 24 hours 10 mM KCl and *Nitzschia* combination treatment (1151 ± 483 SD), the 24 hours KCl treatment (1070 ± 564 SD), the 12 hours KCl treatment (1023 ± 443 SD), and the 24 hours control (584 ± 322 SD).

Table 3.4 Estimated slopes and intercepts for each treatment, with 95% confidence intervals in brackets, that had been back-transformed from the log to the original scale.

Exposure	Intercept	Slope
12 h control	323.754 (316.705; 330.960)	1.0239 (1.0236; 1.0243)
24 h control	374.437 (362.823; 386.422)	1.0089 (1.0084; 1.0095)
12 h KCl	1722.560 (1678.634; 1767.635)	0.9925 (0.9920; 0.9930)
24 h KCl	1018.593 (991.907; 1045.997)	1.0023 (1.0018; 1.0028)
24 h KCl and <i>Nitzschia</i>	2022.009 (1972.395; 2072.872)	0.9947 (0.9943; 0.9952)

*For a power function slope, values > 1 represent positive relationships and slopes < 1 represent negative relationships.

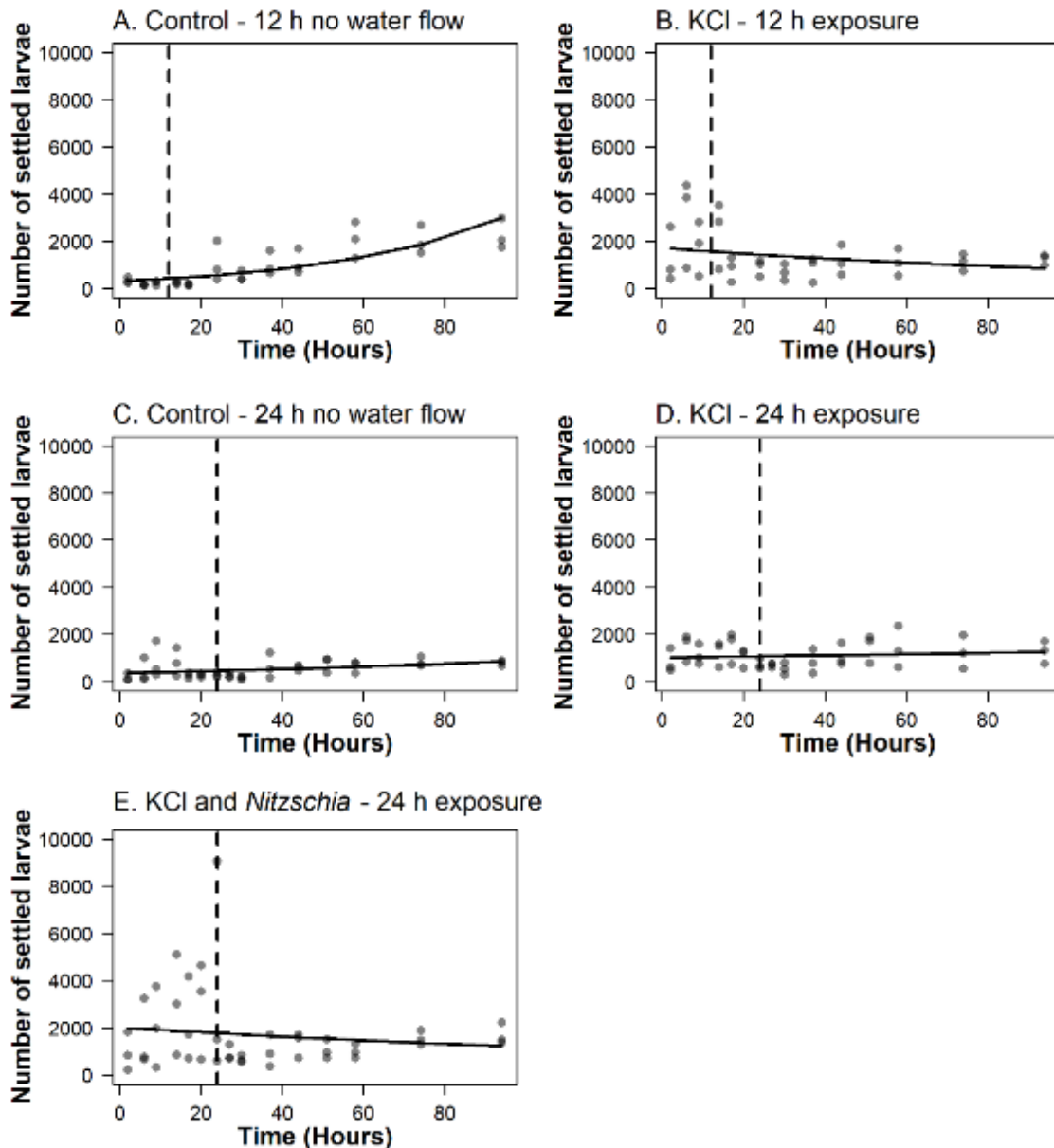


Figure 3.2 Number of settled abalone (*Haliotis midae*) larvae during and post-exposure for 12 hr to 10 mM KCl, 24 hr to KCl, and exposure to a combination of 10 mM KCl and *Nitzschia* for 24 hr with control treatment for each exposure time. The vertical dashed line indicates exposure period for each treatment and no water flows for each control treatment.

At the end of the experiment (94 h), there were still 0.07 ± 0.07 larvae.mL⁻¹ larvae swimming in the 10 mM KCl 12 hours and 0.13 ± 0.07 larvae.mL⁻¹ in 10 mM KCl 24 hours treatment. Most larvae from all treatments had also metamorphosed, but the proportion of metamorphosed larvae differed significantly among treatments ($\chi^2 = 2761.7$, df = 4, $p < 0.0001$). The Tukey

HSD pairwise test shows that all treatments have a significantly higher proportion of metamorphosis than the two controls (Table 3.5). While there was no difference between 2 h 10 mM KCl and 24 h 10 mM KCl and *Nitzschia*, the difference between 24 h 10 mM KCl and 24 h 10 mM KCl and *Nitzschia* treatment was very weak. However, it is important to note that significantly lower metamorphosis in the controls may be due to three cases where low metamorphosis occurred on only one of the two sheets in the tank – one replicate of the 12 h control and two replicates of the 24 h control.

Table 3.5 Pairwise comparison of metamorphosis variation across settlement cue treatments and the controls.

Pairwise comparison	Estimate	Std. Error	z value	P
Control 24h, Control 12h	-0.745	0.031	-24.077	<0.001
KCl and <i>Nitzschia</i> 24 h, Control 12h	0.757	0.034	22.248	<0.001
KCl 12h, Control 12h	0.742	0.038	19.492	<0.001
KCL 24h, Control 12h	0.640	0.034	18.681	<0.001
KCl and <i>Nitzschia</i> 24 h, Control 24h	1.502	0.040	37.211	<0.001
KCl 12h, Control 24h	1.487	0.044	33.938	<0.001
KCL 24h, Control 24h	1.385	0.041	34.147	<0.001
KCl 12h, KCl and <i>Nitzschia</i> 24 h	-0.016	0.046	-0.343	0.997
KCL 24h, KCl and <i>Nitzschia</i> 24 h	-0.117	0.043	-2.729	0.048
KCL 24h, KCl 12h	-0.101	0.046	-2.195	0.175

iii. *Larval settlement in response to KCl and free-swimming Nitzschia sp. after short exposure*

Table 3.6 Estimated slopes and intercepts for each treatment, with 95% confidence intervals in brackets, that had been back-transformed from the log to the original scale.

Treatment		Intercept	Slope*
Cue	Substrate		
Control	Blank	4.698 (3.445; 6.406)	0.9949 (0.9867; 1.0031)
	Diatoms	69.877 (50.879; 95.969)	1.0170 (1.0086; 1.026)
Reference control	Diatoms	101.125 (73.778; 138.608)	1.0153 (1.0068; 1.0237)
	Diatoms	103.588 (75.539; 142.053)	1.0087 (1.0003; 1.0172)
KCl	Blank	5.276 (3.442; 8.088)	0.9945 (0.9833; 1.0059)
	Diatoms	157.316 (114.939; 215.316)	1.0095 (1.0012; 1.0180)
<i>Nitzschia</i>	Blank	3.699 (2.356; 5.806)	1.0029 (0.9916; 1.0144)
	Diatoms	121.986 (89.065; 167.075)	1.0139 (1.0056; 1.0224)
KCl and <i>Nitzschia</i>	Blank	3.976 (2.538; 6.228)	0.9996 (0.9881; 1.0112)
	Diatoms	137.868 (100.693; 188.768)	1.0115 (1.0031; 1.0199)

*For a power function, slope values > 1 represent positive relationships and slopes < 1 represent negative relationships. If 95% confidence intervals of a slope estimate overlap 1, the relationship is insignificant.

The larval settlement was negligible on blank sheets in all treatments and did not change over time (averaged over all samples 4 ± 4 SD, Tables 4.1 and 4.5). On diatom-coated sheets, larval settlement differed significantly among the different treatments (Tables 3.2 and 3.6). Initially, the larval settlement was higher in a combined 10 mM KCl and *Nitzschia* treatment and 10 mM KCl-only treatment, while the control treatment had the lowest larval settlement, but these differences were not as pronounced as in the other experiments (see, e.g., the intercepts in Table 3.6). The settlement on diatom-coated sheets increased over time in all treatments and controls (Fig. 3.3 and the slope estimates in Table 3.6).

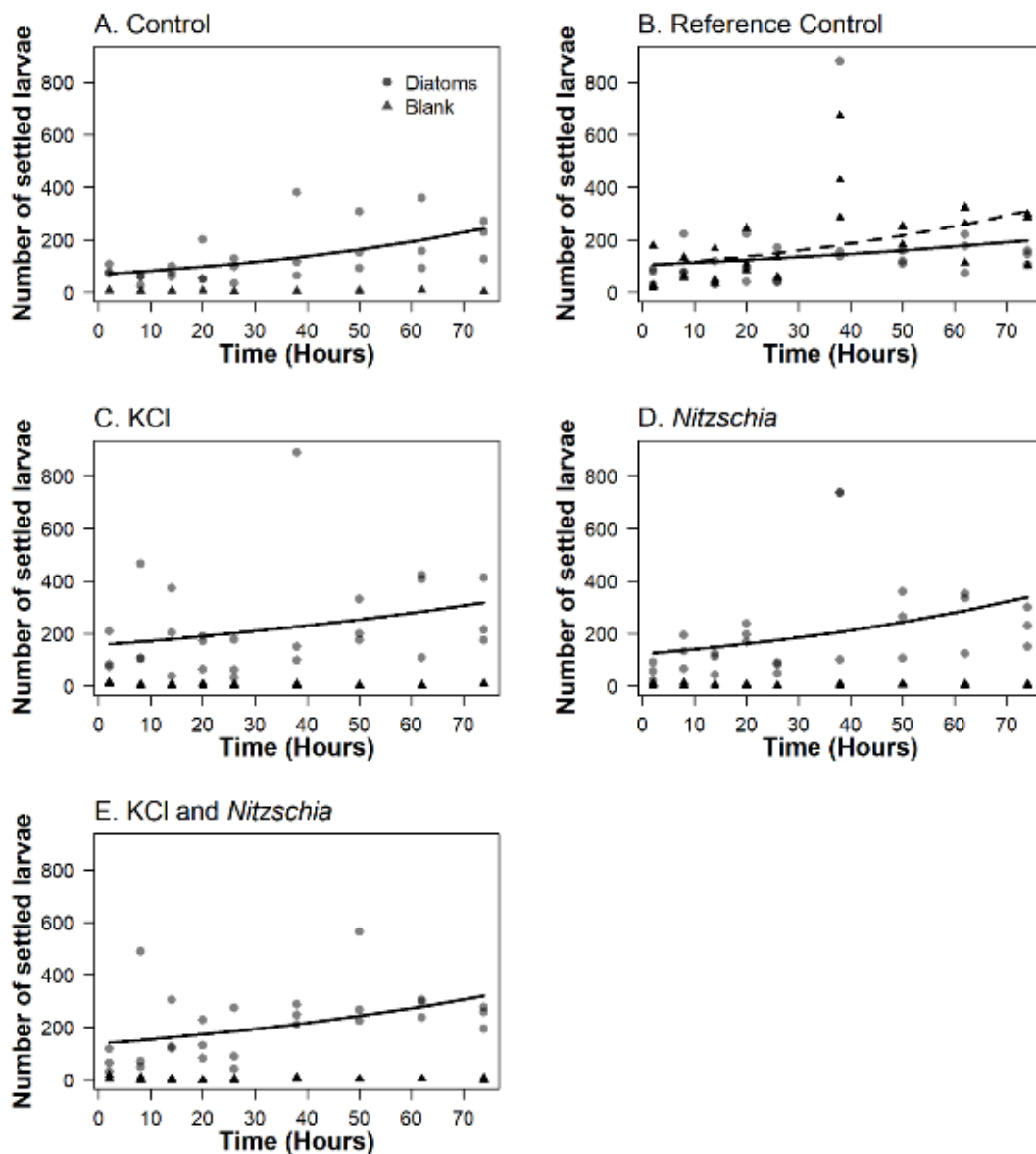


Figure 3.3 Number of settled abalone (*Haliotis midae*) larval after 60 min exposure to three settlement cues (10 mM KCl, *Nitzschia* sp, a combination of 10 mM KCl and *Nitzschia*) and a control (no *Nitzschia* or KCl) and reference treatment (10 mM KCl and *Nitzschia* exposed for 24 hr) on diatoms coated sheets.

Larvae settled on the aquarium surface were calculated at the end of the experiment, and there was no difference (per.m^{-2}) between treatments. More larvae settled on the aquarium's surface than on the settlement sheet, either coated or uncoated. At the end of the experiment, the percentage of larvae swimming in the water column was not different between treatments. None of the few larvae that had settled on the blank sheets had metamorphosed; therefore, the statistical test was done only on the diatom-coated sheets. Although almost all larvae (> 95.8% in all replicates) had metamorphosed on the diatom-coated sheets, a weak significant difference in metamorphosis was found among different treatments ($\chi^2 = 11.6$, $df = 4$, $p = 0.02$). However, a Tukey HSD test found no significant pairwise differences between treatments or controls (all p values > 0.057) (Table 3.7). Therefore, the significant ANCOVA p -value is not biologically meaningful and could possibly be a *type I error*.

Table 3.7 Pairwise comparison of metamorphosis variation across settlement cues on diatom-coated sheet treatments and the controls

Pairwise comparison	Estimate	Std. Error	z value	P
KCl, Control	-0.018	0.122	-0.151	1.000
Diatom & KCl, Control	-0.182	0.115	-1.589	0.499
Diatoms only, Control	-0.199	0.115	-1.727	0.412
Control, Reference control	-0.270	0.106	-2.561	0.076
KCl, Reference control	-0.252	0.095	-2.665	0.058
Diatom & KCl, Reference control	-0.088	0.086	-1.032	0.838
Diatoms only, Reference control	-0.071	0.086	-0.824	0.922
Diatoms & KCl, KCl	-0.164	0.105	-1.565	0.514
Diatoms only, KCl	-0.181	0.105	-1.716	0.419
Diatoms only, Diatoms & KCl	-0.017	0.097	-0.175	1.000

3.4 Discussion

In the natural environment, competent larvae of marine gastropods tend to swim downward instinctively to explore the substrate by randomly testing the contacted surface. If the substrate is suitable, they will attach; otherwise, they will swim off in search of a suitable substrate for their future development and growing needs (Li et al. 2006). Larval settlement is highly influenced by chemical cues, the characteristic of a suitable environment for newly recruited individuals (Huggett et al. 2005). Moss and Tong (1992a) reported that inducers such as synthetic chemicals (KCl and GABA) arrest the movement of the cilia of the veliger, forcing the abalone larva to move downwards. Chemical cues such as potassium chloride, dopamine, and glutamine can down-regulate larval swimming and induce early attachment and metamorphosis of *Haliotis iris* (Alfaro et al. 2014). Although the mechanism by which KCl

induces settlement of abalone larvae is poorly understood, Baloun and Morse (1984) reported that an increase in the concentration of K^+ in a defined seawater medium induces settlement in larvae *Haliotis rufrescens*. However, the first experiment in the current study showed that exposure to 10 mM KCl induced three times higher larval settlements than the higher concentrations (15 and 20 mM), suggesting that there is an optimal concentration of KCl to induce settlement. Li et al. (2006) reported a similar trend that 10 mM KCl induced settlement of *H. diversicolor*, and high concentration of KCl (20 mM) were toxic to the larvae, resulting in a high level of mortality. Like Li et al. (2006), high concentrations of KCl might have been toxic to the larvae and hindered settlement in the current study. In contrast, Bryan and Qian (1998) showed that the percentages of settled *H. diversicolor* larvae were not different between 10 and 20 mM of KCl. The different response from Bryan and Qian (1998) and this study suggests that the same species might respond differently to chemical cues.

Continuous exposure of *H. rufrescens* larvae to 10 mM K^+ increased the settlement (Yool et al. 1986). It was hypothesized that more prolonged exposure of *H. midae* larvae to settlement cues (KCl and *Nitzschia*) would maximize settlement and metamorphosis compared to larvae subjected to shorter exposure. However, in the second experiment, exposure of larvae to 10 mM KCl for 12 hours displayed higher overall settlement than the number of settled larvae exposed to 10 mM KCl for 24 hours and the control treatments. The difference between 12 hours of KCl and 24 hours of KCl larvae exposure may be due to the difference in water quality between the treatments. Larvae (*Haliotis laevigata*) are sensitive to poor water quality (Hindrum et al. 2001). Poor water quality stresses the abalone and slows its growth; in the worst case, water quality can deteriorate to the extent of causing mortalities (Hindrum et al. 2001). Therefore, stopping water flow for extended periods might have negatively affected the water quality to a point where it affected settlement. However, more prolonged exposure (24 hours) to the combination of KCl and *Nitzschia* resulted in the highest larval settlement compared to all other treatments. Microalgae use carbon dioxide (produced by larvae through respiration) during photosynthesis, where oxygen is produced, and it absorbs ammonium and nitrate in the water during assimilation (Abdel-Rouf 2012). The planktonic microalgae (*Nitzschia*) might have helped maintain good water quality in this treatment, resulting in the highest settlement. The settlement almost doubled in the combination of 10 mM KCl and *Nitzschia* exposed for 24 hours of treatment compared to KCl exposed for 12 hours during the exposure. One possible reason is that after 24 hours post-exposure, *H. midae* larvae were more developed, which might have contributed to the settlement. Barlow (1990) noticed rapid

settlement of *H. rufescens* in response to exposure to GABA in older larvae. Moss and Tong (1992b) also reported that settlement was more significant for older larvae when compared to younger larvae. Another possibility is that combining two settlement cues, KCl and *Nitzschia*, is more likely to result in a higher settlement than a single cue. Potassium chloride is known to down-regulate abalone swimming and force the larvae to settle (Moss & Tong 1992a, Alfaro et al. 2014), while *Nitzschia* releases aspartic acid, which strongly induces the attachment of swimming abalone larvae (Gordon et al. 2004). Similar results were reported by Li et al. (2006), showing that combining 10 mM KCl and natural diatoms could increase settlement by 48% compared to 28% for the control (natural diatoms) treatment.

Post-exposure settlement in the second experiment treatments showed a significant reduction in the mean number of settled larvae (Fig. 3.2), as was observed in the first experiment (Fig. 3.1). Barlow (1990) reported that at low concentrations, GABA does not paralyze cilia which cause settlement in larvae. Assuming a similar response occurs in larvae exposed to low concentrations of KCl, in this experiment, the water-flow post-exposure period diluted the concentration of KCl in the water, and larvae that had not metamorphosed could swim off the substrate. These results are supported by Morse (1991), who stated that it took up to twenty hours for larval *H. rufescens* to shed its velum (the fringe of ciliated cells surrounding its head that allows it to swim). Similarly, Bryan and Qian (1998) reported that it takes up to 48 hours for larval *H. diversicolor* to start metamorphosis after initial settlement. Therefore, it is likely that *H. midae* larvae had not yet shed their velum and, therefore, not metamorphosed by the end of the 6-, 12-, and 24-hour exposure periods, enabling them to swim off the settlement sheet when KCl concentration was reduced by flushing. The mean number of settled larvae in treatments with KCl was less than the control treatment at the end of the experiment, indicating that not all larvae in the KCl treatment were reattached to the settlement sheet after the initial settlement.

In the first and second experiments, the settlement of larvae in the control treatments increased over time and could sometimes even surpass settlement compared to other treatments post-exposure. Barlow (1990) reported that unstimulated larvae spend less time swimming as they age. These results suggest that prolonged exposure (24 hours) of *H. midae* larvae to either chemical cues (KCl) or a combination of chemical cues and biological (*Nitzschia*) before release may not increase settlement in the ocean as the inducers are primarily effective when KCl concentration levels are 10 mM. The abalone larvae may also settle on the surface of the bucket before seeding during the hours of exposure.

Results from the third experiment showed that settled larvae were initially higher for the reference control treatment (combination of 10 mM KCl and *Nitzschia* exposed for 24 hours) compared to the diatom-coated sheets in the short exposure control. However, the short exposure control treatment had a higher rate of settlement over time (greater slope value) on the diatom-coated sheets compared to the reference control (Table 3.6). Based on these results, the exposure period hypothesis was therefore rejected.

Settlement is affected by several factors, including biological cues associated with the substrate, which are considered the primary stimuli for initiating larval settlement in many marine molluscs (Li et al. 2006). Larvae must contact the substrate to be exposed to a biological cue that triggers settlement (Slattery 1987). The larval settlement differed between the treatments of diatom-coated settlement sheets. Daume et al. (1999b) reported that diatoms induce settlement for abalone larvae *Haliotis rubra*, which is supported by Li et al. (2006), who showed that diatom films (with both mixed diatoms and bacteria) had better larval settlement than those exposed to uncoated sheets. Although the short exposure treatments were statistically different from the control, the treatments almost appear similar in a graph, suggesting that the difference was not big enough to have practical application for field-based studies or the hatchery. Additionally, the settlement was not different between treatments and the control on uncoated sheets.

Specific environmental stimuli are required to initiate the metamorphosis of many marine invertebrate larvae (Yool et al. 1986). Once larvae find a suitable substrate to settle, metamorphosis follows shortly after, an irreversible commitment to the benthic mode of life (Morse 1991). The mean number of larvae reaching metamorphosis was more significant in the treatments compared to the controls for experiment 2 (Tables 4 and 6). At the same time, no meaningful difference was found between the treatments and the controls for experiment 3. For all replicates in experiment 3, more than 95% of settled larvae had metamorphosed. However, it is essential to note that for the second experiment, the lower proportions of metamorphosis in the controls are due to low metamorphosis (< 17% metamorphosed) on three specific sheets – one 12h control replicate and two 24h control replicates. In each case, the rate of metamorphosis was high on the other sheet in the pair. On all other sheets, more than 95% of larvae had metamorphosed in the controls and treatments, similar to experiment iii. The poor metamorphosis may be due to different layers (thin or thick) of diatoms, which affect settlement (S. Ashlin Wild Coast Abalone farm-hatchery manager, pers.comm). Gapasin and Polohan (2004), Kang et al. (2004), and Li et al. (2006) reported that after continuous exposure of larvae

to KCl, metamorphosis was induced. Metamorphosis begins 48 hours post-settlement (Morse 1991, Bryan & Qian 1998). In the second and third experiments, larvae were exposed to the chemical (KCl) and the biological (*Nitzschia*) inducers for 12 hours, 24 hours, and one hour before being flushed out. Therefore, metamorphosis in these experiments would appear to have not been influenced by either 10 mM KCl or planktonic *Nitzschia*; instead, the biofilm coating on the settlement sheets may have induced larval metamorphosis. Gordon et al. (2004) reported that layers of diatoms such as *Nitzschia*, *Navicula*, and *Amphora* improved the attachment and settlement of abalone larvae *Haliotis discus hannai*. Maximizing larval metamorphosis is essential for the success of the abalone hatcheries, ranching, and stock enhancement programme; these findings can, therefore, guide abalone farmers and stock enhancement programme managers in the selection and use of appropriate settlement cues that will increase larval settlement and metamorphosis.

Larvae are eventually forced to settle once their endogenous yolk reserve is exhausted, even if they have not yet been exposed to a settlement inducer (Gapasin & Polohan 2004). In the third experiment, the small percentage of larvae (less than 1%) that settled on the uncoated sheet had not yet metamorphosed after 74 hours. Li et al. (2006) reported similar results, showing that 5% of *H. diversicolor* larvae settled on the uncoated surface (with no settlement cues) and did not metamorphose. Gapasin and Polohan (2004) reported that in the absence of settlement cues, exploring larvae will ultimately settle without inductive cues once the endogenous yolk reserve is exhausted. Hence, in this study, some larvae were observed settled on the surface of the aquarium, probably due to the absence of cues to induce the settlement of uncoated sheets.

At the end of the second experiment (94 hours), some larvae were still swimming in both KCl 12-hour and 24-hour exposure treatments. Similarly, in the third experiment, some larvae were still swimming after 72 hours in all treatments. Reasons for this could be the following. (a) The larvae quality varied among studies. The current study was limited to the broodstock supplied at the facility, and the confounding effect related to larval batch quality was regrettably unavoidable. (b) Slight differences in the diatom layer on the settlement sheets may have existed. Different layers (thin or thick layers) of diatoms can affect the settlement of abalone larvae (S. Ashlin, Wild Coast Abalone Farm-hatchery manager, pers.comm). (c) In experiment two, *H. midae* larvae were nine days old at the end of the experiment compared to the eight-day-old larvae in experiment three. Moss and Tong (1992b) reported that the developmental stage of larval affected the settlement of *H. iris*, with higher settlement occurring for older larvae.

The inducer effect of KCl on the settlement and metamorphosis of a variety of marine invertebrate larvae has been widely acknowledged (Yool et al. 1986, Bryan & Qian 1998, Gapasin & Polohan 2004, Kang et al. 2004, Li et al. 2006, Yu et al. 2010). However, the results from this study differ from those of other studies. One possible reason for the inconsistencies could be that *Haliotis* spp. behave differently, in that what is effective for one species may or may not necessarily trigger the same response for others (Yu et al. 2010). Another possible reason is that the current study used a commercial hatchery larval stocking density (2 larvae.mL⁻¹), a typical density used in South African *H. midae* hatcheries (S. Ashlin, Wild Coast Abalone farm-hatchery manager, pers. comm). Other studies have, however, used 0.2 larvae.mL⁻¹ (Bryan & Qian 1998, Gapasin & Polohan 2004, Kang et al. 2004, Li et al. 2006, Liu et al. 2006, Yu et al. 2010). Liu et al. (2006) reported that increased rearing density increases the possibility of collision among individuals, causing an abrupt cessation of the beating of the preoral cirri, followed by rapid retraction of the velum. Abalone larvae swim with an extended velum, which, if retracted, results in the larvae being unable to swim, sinking to the aquarium's bottom (Alfaro et al. 2014). This reduces the chances of the larvae settling on the suspended settlement sheets. Finally, inconsistencies between the current study's findings and those of previous studies may result from slight differences in experimental design. In the studies of Bryan and Qian (1998), Gapasin and Polohan (2004), and Li et al. (2006), abalone larvae were exposed to the chemical inducer for ± 5 days with a partial dilution of water once a day in some experiments or none at all in others. In this study, *H. midae* larvae were exposed to the chemical and biological inducer for varying periods (1, 6, 12, and 24 hours), and water flow was resumed post-exposure. Morse (1991) reported that abalone larvae must contact the inducer substrate. In this experiment, flushing out of the KCl post-exposure significantly affected larval settlement, which may be why the settlement was reduced post-exposure, and the results are different from others with continuous exposure.

In conclusion, longer exposure (+12 hours) of *H. midae* larvae to 10 mM KCl and *Nitzschia* enhanced settlement. This suggests that continuous exposure of larvae to an inducer until metamorphosis, which could be achieved in a hatchery but not in the ocean, will increase settlement. These results also showed a dramatic decrease in settlement of *H. midae* larvae post +12 hours exposure, while 60 minutes exposure to KCl and *Nitzschia* did not result in a settlement difference that was big enough to have practical application in the field-based studies or the hatchery. These results suggest that exposure of *H. midae* larvae to biological (*Nitzschia*) and chemical cues (10 mM KCl) for 24 hours or less before release may not increase

settlement in the ocean. The inducers are primarily effective when KCl concentration levels equal 10 mM. Therefore, these results cannot draw a biological conclusion for field studies due to poor settlement when the biological cues are not highly concentrated ($120 \text{ cells.mL}^{-1}$), and KCl levels in seawater are typically below 10 mM (Todd et al. 1991), and it does not improve settlement without adding extra KCl in the seawater.



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CHAPTER 4

Staining of larval abalone (*Haliotis midae*) shells using two epifluorescent dyes

4.1 Introduction

The South African wild abalone (*Haliotis midae*) fishery has experienced a considerable decline in population abundance over the last three decades, primarily owing to heavy fishing pressure from the illegal catch (Witte 2017). Two techniques have been evaluated to restore depleted stocks of this commercially valuable species. Stock enhancement using cultured juveniles has been practiced in Japan for several decades, while in South Africa, there are three abalone ranching operations on a commercial scale that have been operating for a couple of years. The associated high cost of seeding juvenile abalone has led to an interest in the possibility of larval seeding (Preece et al. 1997). Studies on the larval seeding of abalone have been conducted on a small-scale, experimental level (Tong et al. 1987, Preece et al. 1997, Chick 2010a, Mills-Orcutt et al. 2020) on various species. Results have shown high variation in survival for the different species seeded, which is likely influenced by the different seeding methods. This suggests that strategies for seeding larvae in the wild are probably site and species-specific.

When conducting field-based studies or stock enhancement programmes, released individuals must be unambiguously identifiable from wild animals, and therefore, a reliable, effective tagging method is required. However, tagging methods to identify individuals uniquely can often be problematic owing to extensive handling, which is also labour-intensive if a large number is tagged and can also negatively affect the survival and growth of the individuals (Chick, 2010a). Free swimming abalone larvae are small in size (*H. midae*: $207 \times 265 \mu\text{m}$) (Genade et al. 1988), which makes it challenging to tag them individually, and larvae are also sensitive to handling and manipulation (Chick 2010a). The batch-tagging method is an effective method for tagging many individuals simultaneously. The marking tag can either be added to water, where the animals are marked by immersion, or incorporated into the feed (Chick 2010a). The immersion marking method is the most suitable for small life stages

(Purcell & Blockmans 2009), such as abalone larvae, since they may not begin feeding until settlement.

Fluorochromes are calcium-binding substances that are incorporated at the site of active mineralization of bone, known as calcification front at the site of new bone formation (Rahn & Perren 1971, Stuart & Smith 1992, Day et al. 1995, Kaehler & McQuaid 1999, Moran 2000). After binding, the dye provides a bright epifluorescence reference, which remains embedded in the growing shell at the top of the spire (Chick 2010a). Fluorochromes dyes have been used for batch-tagging small life stages such as class Gastropoda: abalone larvae (Day et al. 1995, Chick 2010a), snails (Moran 2000) and class Bivalvia (van der Geest et al. 2011). Studies have shown that fluorochrome dye does not appear to have a detrimental effect on aquatic larvae when administered at an appropriate concentration (Day et al. 1995, Moran 2000, Uggeri et al. 2004, Moran & Marko 2005) and extended immersion periods at the appropriate concentration can result in higher levels of epifluorescence brightness (Purcell & Blockmans 2009). However, fluorochrome dyes can be toxic in marine animals if used in excessively high concentrations for prolonged periods (Purcell & Blockmans 2009). Fluorochromes commonly used for marking the shell of marine species include calcein, xylanol orange, alizarin red S, oxytetracycline, and tetracycline. Fluorochrome calcein is a nonfluorescent, cell-permeant compound converted by intracellular esterases into calcein, the anionic fluorescent form that fluoresces bright green when combined with calcium (Uggeri et al. 2004). Calcein has been used successfully to mark the shell of different species in numerous growth studies, i.e., abalone larval, *Haliotis rubra* (Day et al. 1995, Chick 2010a), juvenile scallop, *Amusium balloti* (Lucas et al. 2008), green-lipped mussel larvae, *Perna canaliculus* (Fitzpatrick et al. 2013); and the bivalve, *Loripes lacteus* (van der Geest et al. 2011). The calcein mark retained within the shell during settlement and metamorphosis remains embedded in the growing shell at the top of the spire (Collin and Voltzow, 1998). Xylanol orange is fixed in newly formed calcified tissues where it remains throughout the growth of the animal (Rahn & Perren 1971) and has been successfully used to mark abalone larvae, *H. rubra* (Day et al. 1995).

The release of hatchery-produced larvae has been identified as an alternative option to building depleted wild abalone stock; millions of larvae can be produced in a hatchery on demand and cost-effectively. However, research still needs to be done to test the viability of using abalone larvae in terms of potential economic benefit, including tracking the survival and growth of released larvae. One of the critical factors required to determine the feasibility of this stock enhancement technique is the unambiguous identification of the released abalone from the wild

stock. Marked recapture allows quantification of fundamental aspects of biology such as mortality, growth, population size, age at maturity, and possible track movement, which is essential information for stock enhancement programmes (Lucas et al. 2008). Identifying hatchery-produced larvae will also enable proof of resource ownership for abalone ranching programmes, which is also critical for the further development of abalone ranching. This study aimed to compare and identify an ideal dosage of two fluorochrome dyes (calcein and xylene orange) in staining the shell of abalone (*H. midae*) larvae and assessing their effect on initial survival and settlement as well as brightness and longevity of resultant mark. The objectives of this study were to determine (1) whether the concentration of the chemical dyes has any effect on larval survival and settlement and (2) if the combination of immersion time and epifluorescence dye concentrations can induce a persistent marker in the larval shell which is detectable using fluorescent light. The results from this study could be used in abalone larvae reseedling or stock-enhancement programmes to help identify the hatchery-produced juveniles upon recapture.

4.2 Material and methods

Fertilized abalone (*H. midae*) eggs were collected on a hatching tray and hatched in 1 µm filtered seawater maintained at 17°C. Newly hatched larvae (veliger stage) are free-swimming and swim over into the larval rearing tank (LRT), with a flow-through system and each LRT received independent aeration and filtered (1 µm) UV-treated seawater. Larval development was determined by taking triplicate 10 mL water samples from each LRT and identifying the larval developmental stage under a dissecting microscope (NSZ-606, Best Scope). Distinctive external and internal larval features were identified following descriptions by Genade et al. (1988) (Table 4.1). A developmental stage was considered to be attained when more than 50% of the larvae observed in a sample had the features associated with that stage.

The study was conducted at Wild Coast Abalone Farm (Pty) Ltd (32°45'05'S 28°16'27' E), South Africa hatchery. Larvae were developed until they reached a stage where they could settle, six days after fertilization.



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Table 4.1 Developmental stages of abalone larvae *Haliotis midae* (Genade et al. 1988)

No.	Life cycle stage	Developmental stage	Description
1	Egg	Fertilization	Fertilization and discharge of the two polar bodies
2	Trochophore	Hatching	Initial larval shell secretion
3	Veliger	Pre-eye spot	Appearance of the pre-eye spot
4	Veliger	Retracted velum	Development of a short spine on the cephalic tentacle
5	Veliger	Crawling and settling	Development of the fourth tubule on the cephalic tentacle

Experimental Setup

The experiment was conducted in 20 L tanks filled with 1 μm filtered seawater mixed with sodium bicarbonate (0.1 g. L⁻¹) to buffer the pH in the seawater. Calcein-AM (Sigma-Aldrich, Johannesburg, South Africa) and xylene orange tetrasodium salt (Reflecta Laboratory Supplies, Johannesburg, South Africa) were used as fluorescent markers, with each at a concentration of either 25 mg. L⁻¹ or 50 mg. L⁻¹. The chemical powder was dissolved in the water before adding the abalone larvae. Approximately 40,000 six-day-old larvae that were competent to settle were collected from the larval rearing tanks onto an 80 μm screen and transferred into the tanks (2 larvae mL⁻¹) containing calcein or xylene orange. Larvae were immersed in the marking baths for 24 h and 48 h, with both treatments starting at the same time (t 0), and then held for their respective immersion times (24 h and 48 h). Larvae were held in each tank, with aeration but without water flow. Two controls containing no fluorochrome dye for the 24 h and 48 h periods were included. All treatments had three replicates. The tanks were in a temperature-controlled room maintained at $18 \pm 0.5^\circ\text{C}$.

i. Evaluating the effect of calcein and xylene orange immersion period on abalone larval survival

Immediately after the respective immersion periods, patches of dead larvae, which tend to accumulate in dark clumps at the bottom of the rearing tank (referred to in abalone hatcheries as "spotting"), were siphoned from each tank. The remaining larvae were gently poured through an 80 μm screen and placed into a tank with flow-through water to remove excess dye. Once clean, the larvae were gently poured into 10 L tanks containing filtered (1 μm) seawater. Tanks were connected to a flow-through system, with the water exiting via a banjo sieve covered with

80 μm mesh. Each tank receives independent aeration and filtered (1 μm) UV-treated seawater (6 L.h⁻¹). The photoperiod was set at 12L:12D. Ambient temperature was controlled with air conditioners and maintained at 16°C. A water chiller (Titan 2000, Aqua Medic) was used to maintain a suitable temperature in the aquariums. Throughout the experiments, the temperature in each aquarium was monitored daily (using a 0.1°C gradient thermometer). Two plastic sheets (15 × 15 cm = 900 cm² total surface area per tank) that were placed in outside tanks supplied with incoming seawater and allowed to develop a film of natural diatoms were placed in each tank.

Post-exposure larval survival was determined by taking 10 mL water samples from each tank in triplicate. The number of swimming larvae was counted under a dissecting microscope (Best Scope, NSZ-606) as a function of the initial stocking density (2 larvae.mL⁻¹).

ii. *Determining the effect of calcein and xylenol orange immersion period on abalone larvae initial settlement*

To determine the effect of calcein and xylenol orange on the settlement of larvae at different concentrations and immersion times, after 24 and 48 h post-transfer to the clean 10 L tank, each settlement sheet was briefly removed from each aquarium and photographed (Canon camera EOS 2000D) at high resolution (≈ 24.1 megapixels). The number of larvae settled was counted on both sides of each sheet using DotDotGoose software (version 1.1.1, Ersts 2019). The experiment ended after 48 h because the settlement sheet was small and insufficient to provide food for an extended period. The data presented was recorded after 48 h and 24 hours post 24- and 48-h immersion periods, respectively, to ensure that larvae were in the same developmental stage.

iii. *Determining the persistence of epifluorescent dose in the shell of juvenile H. midae*

The experiment was conducted following the same method described in the first experiment to determine the persistence of calcein and xylenol orange stain in the shell of juvenile *H. midae*. After the respective immersion period, 3 500 and 4 500 larvae were placed into small (26 cm²) and large (31.8 cm²) abalone settlement bags, respectively. Bags were randomly placed in the hatchery larval culture room with two replicates for each treatment. The inside of the bags was coated by filling the plastic bag with seawater and allowing a natural diatom film to attach and grow in the bags for two weeks before the experiment. The stained larvae were then introduced into the bags, settling and feeding on the diatom layer. During the first five days of settlement,

the water flow was switched off to avoid flushing the swimming larvae out of the bags. Each bag received aeration while the temperature was maintained at $18 \pm 0.2^{\circ}\text{C}$. Larvae were raised in the settlement bags until food became limiting at 51 days after transfer into the bag. The persistence and level of epifluorescence were determined by taking 15 individuals from each bag at t0 (start of the experiment), and subsequent samples were collected after 24 days, 38 days, 47 days, and 51 days post-transfer to the bags. Samples were stored at -20°C , and after thawing, the shell of each juvenile was observed under a fluorescence microscope (Olympus BX-61) using the FITC set (green fluorescent), DAPI set (blue fluorescent) and Red set (Red fluorescent) to visualize the larval shell. Images of the abalone shell were captured using a Leica DFC 490 camera.

Images were then analyzed for brightness, where the intensity of fluorochrome (brightness of the band) was quantified using "ImageJ" image processing software's freehand selection tool and histogram analysis function (developed by Wayne Rasband at the National Institutes of Health (NIH)). This function quantifies the brightness of each pixel within the selected area. The number of pixels for each value is plotted on a histogram, and the overall mean brightness of the selected area is established. The mean brightness was recorded to measure the total chemical fluorescence for each abalone's shell band (Fitzpatrick et al. 2010).

Statistical analysis

All data were analyzed to check if they met parametric test assumptions using the Shapiro–Wilk test for normality and Levene's test for homogeneity. Levene's test revealed non-homogeneity of variances, and the data were log-transformed to test the effect of different concentrations of the fluorochrome dyes on larval survival and initial settlement. Then, the data were analyzed using Welch's analysis of variance (ANOVA), which is robust to non-equal variance among treatments. The mean epifluorescence brightness between fluorochrome (exposure period and different concentrations) met the parametric test assumption and was analyzed using a one-way ANOVA. For all data, when significant differences ($p < .05$) were found, Tukey's multiple comparisons test was used to determine specific differences among treatments. An independent sample t-test was also used to compare the overall mean between exposure period treatments. The IBM SPSS version 26 statistical programme was used for all data analyses, with all data presented as mean $\pm$ SE.

4.3 Results

i. *Evaluating the effect of calcein and xylenol orange immersion period on abalone larval survival*

Survival of *H. midae* larvae was significantly affected by both immersion periods and different concentrations of fluorochromes. Abalone larvae immersed for 24 hours had an overall higher percentage survival than those exposed to 48 hours of immersion ($t(28) = 7.96$, $p < 0.001$). After 24 h immersion, the highest percentage survival of $86.78 \pm 4.83\%$ and $80.65 \pm 10.54\%$ was recorded in the xylenol orange 50 mg L^{-1} and 25 mg L^{-1} treatments, respectively. The overall survival of abalone larvae declined after 48 h immersion for both fluorescent marker treatments, with survival below 35%. The highest percentage survival of $32.54 \pm 1.17\%$ and $23.73 \pm 1.50\%$ were recorded for xylenol orange 25 mg L^{-1} and calcein 50 mg L^{-1} , respectively, while xylenol orange 50 mg L^{-1} had the lowest survival ($2.35 \pm 0.98\%$) compared to the other treatments, including the control (Fig. 4.2).

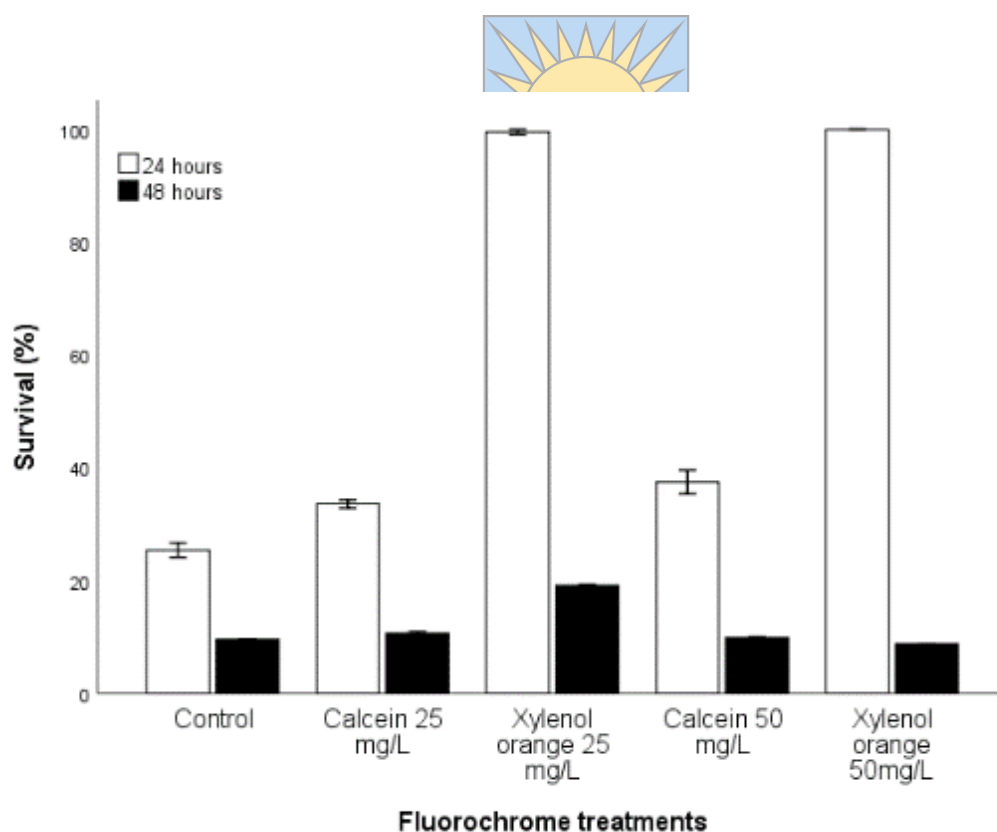


Figure 4.2 Percentage survival ($\pm$ SE) of *Haliotis midae* larvae immediately after 24 and 48 h immersion periods in calcein and xylenol orange at concentrations of 25 mg. L^{-1} and 50 mg. L^{-1} .

ii. **Determining the effect of calcein and xylenol orange immersion period on abalone larvae initial settlement**

The initial settlement of abalone larvae was significantly affected by the immersion period and different concentrations of fluorochrome dyes. The immersion period had a significant effect on the percentage settlement of *H. midae* larvae, with the overall higher percentage of settled larvae recorded (averaged across fluorescent marker type) after 48 h immersion ($5.89 \pm 0.58\%$) compared to 24 h immersion ($2.25 \pm 0.55\%$) ($t(78) = -4.45$, $p < 0.001$). After 24 h immersion, there was no difference in the larval percentage settlement. After the 48-hour immersion period, the percentage settlement of larvae was different between treatments ($F(4) = 46.02$, $p < 0.001$), the xylenol orange 50 mg.L^{-1} treatment had the highest percentage settlement, followed by calcein 25 and 50 mg.L^{-1} while the control treatment had the lowest settlement (Fig. 4.3)

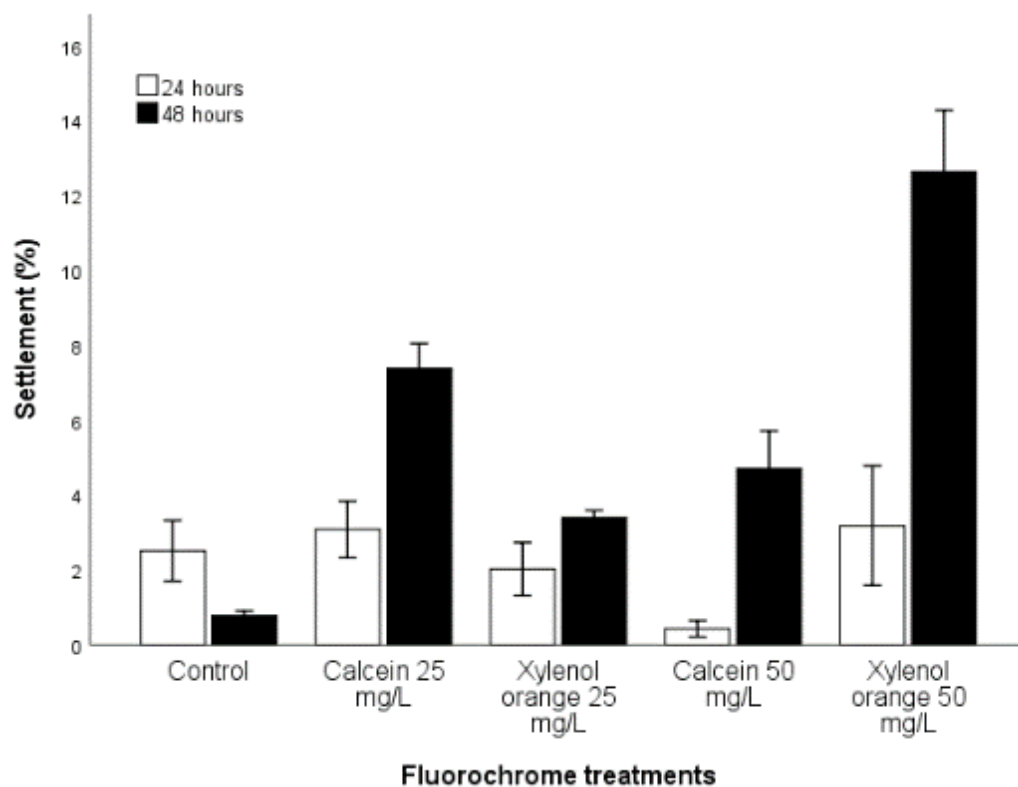


Figure 4.3 Percentage settlement ($\pm$ SE) of *Haliotis midae* larvae after immersed in calcein and xylenol orange at concentrations of 25 mg.L^{-1} and 50 mg.L^{-1} for 24 and 48 h period.

iii. ***Determining the persistence of epifluorescent dose in the shell of juvenile H. midae***

There was a 100% marking success for larvae immersed in the calcein treatment at both concentrations and immersion periods (Fig. 4.4). Epifluorescence bands were evident in all juvenile abalone shells after 51 days. The control treatments also had no natural epifluorescence, and larval shells were not successfully marked in either of the xylenol orange treatments. Higher and more consistent levels of mean brightness were observed in the 50 mg.L⁻¹ calcein batch-tagged juvenile shells, irrespective of the immersion period (F(3)= 137, p < 0.001). The difference in the mean brightness among calcein concentrations varied with immersion time, and 48 h had the highest mean brightness level of both concentrations (t(397)= 2.182, p = 0.030). Larvae immersed for 48 h at 50 mg.L⁻¹ calcein had the highest mean brightness (199.59 ± 1.57 SEM) compared to those immersed for 24 h at 25 mg.L⁻¹ (161.16 ± 1.63 SEM). There was no difference in the level of mean brightness associated with time (decrease of the mean brightness level) of *H. midae* juveniles sampled before and post-settlement (Fig. 4.5).

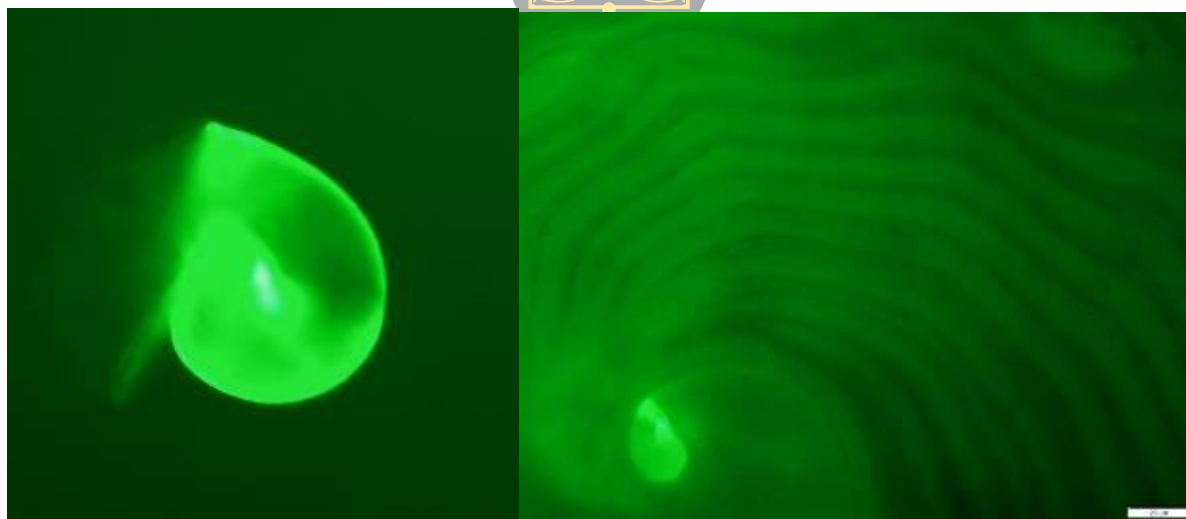


Figure 4.4 Micrograph of a calcein-marked larva of *Haliotis midae* viewed with a fluorescence microscope. (A) 7day-old larvae, immediately prior to settlement (B) 51 day-old stained part of the juvenile shell post settlement.

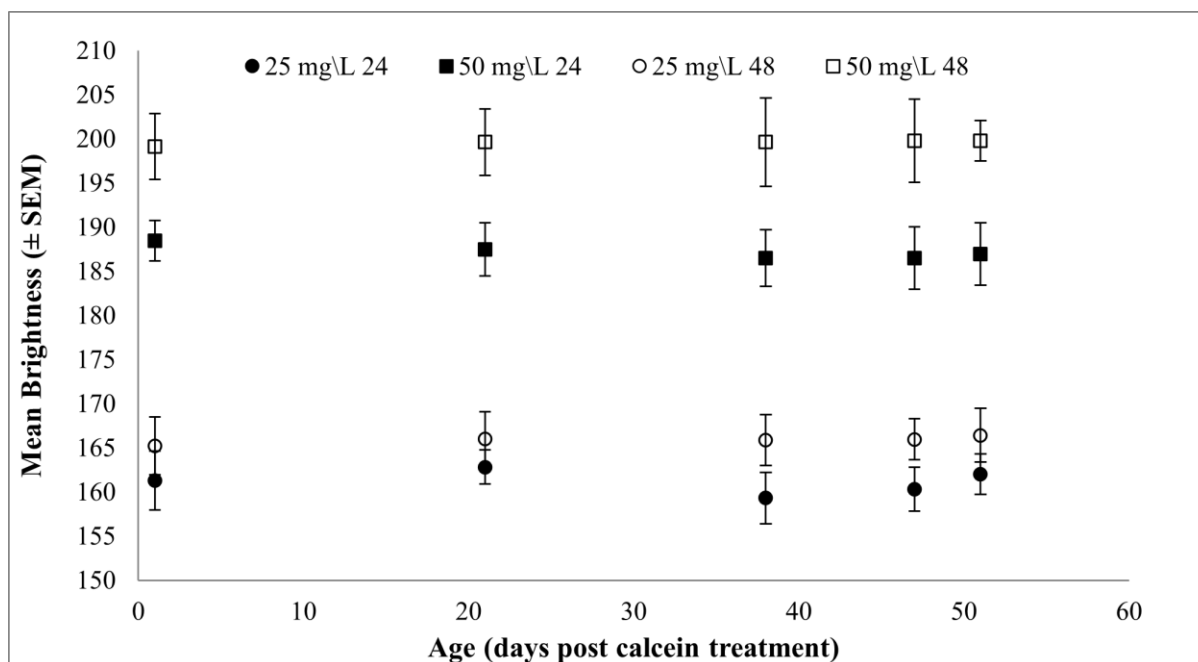


Figure 4.5 Mean brightness ($\pm$ SEM) of fluorochrome on the shell of juvenile *Haliotis midae* immersed for 24 and 48 h at two concentrations (25 and 50 mg L⁻¹) of calcein.

4.4 Discussion

The choice of fluorescent dye for batch-tagging abalone larvae should not be based solely on tag brightness; post-staining survival is also essential. Larval survival is crucial for both abalone hatcheries and stock enhancement programmes. Longer immersion (48 h) of *H. midae* larvae on calcein or xylenol orange affected survival, ranging between 10% and 20% compared to 40% and 95% after 24 h immersion. This survival percentage differed from that found by Chick (2010a), who observed that staining *Haliotis rubra* larvae with calcein does not appear to affect larval survival. These differences could be attributed to (i) different methods used in these studies to determine survival; (Chick 2010a) is based on assumptions while survival was measured in this study. (ii) Species-specific abalone settlement variability may have resulted in some larvae settling on the side surface of the tank after 48 h immersion; hence, fewer larvae were swimming in the water column.

Fluorescent dyes can be toxic to marine organisms (Day et al. 1995) when administered at high concentrations or for extended periods (Purcell & Blockmans 2009). Fluorescent dyes positively affected the survival of *H. midae* larvae, and in both immersion periods, survival was higher in all treatments than in control. Similar results were reported by Moran and Marko (2005), who tested the survival of bivalve larvae *Mytilus trossulus* marked with calcein, which

was significantly higher in 7-day post-marked larvae ($38 \pm 3\%$ (SD)) compared to unmarked larvae ($21 \pm 4\%$ (SD)). The underlying mechanism for this positive effect on survival is unknown. Moran and Marko (2005) stated that one of the significant causes of mortality in larval culture is microbiological contamination, predominantly bacteria and viruses. While microorganism concentration was not evaluated in this study, pausing UV-treated water flow during the staining period might increase the chance for microorganism growth. The positive effect of calcein and xylenol orange on the survival of *H. midae* larvae suggests that both fluorochrome dyes possibly had an antibiotic or antiviral effect.

Abalone larvae must survive the planktonic stage and seek a suitable substrate to change from swimming larvae into benthic juveniles (Leighton 2008). All abalone larvae settled post-immersion across all treatments. However, more extended immersion periods influenced the settlement of *H. midae* larvae, resulting in a higher percentage of settlement. This may have resulted from the stress caused to abalone larvae during the longer (48 h) immersion periods. Larvae (*Haliotis laevis*) are sensitive to poor water quality, which stresses the abalone and slows growth, in the worst case, water quality can deteriorate to the extent of causing mortalities (Hindrum et al. 2001). While Yu et al. (2010) reported that abalone larvae fall out of suspension and consequently attach to the substratum under the stressed condition. After 48 h immersion, *H. midae* were possibly stressed, forcing more larvae to settle. It is possible that abalone larvae were more developed after 48 h immersion, which might have resulted in a higher percentage of settlement (Moss & Tong 1992b).

Different fluorescent dye concentrations also play a vital role in settlement of *H. midae* larvae. While there was no difference between the treatments and the control after 24 h immersion, xylenol orange (50 mg.L^{-1}) and calcein (50 mg.L^{-1}) had higher settlement after 48 h immersion. It has been shown in previous small-scale experiments that abalone larvae can continue to settle for up to 94 hours post-initial settlement (Mzozo et al. 2023). Unfortunately, the percentage settlement of *H. midae* post-settlement was not measured beyond 48 h. It is, therefore, unclear what might have happened with unsettled larvae from the control treatment. Therefore, conclusions on the effect of fluorescent dye on the long-term settlement of *H. midae* larvae should be made with caution.

During the batch-tagging of abalone larvae, the epifluorescence mark is embedded in the spiral of the growing shell of abalone (Chick 2010a). The incorporation of epifluorescent markers in the shell increases with an increase in dye concentration during the fluorescent and immersion

periods (Pirker & Schiel 1993). This study showed that the use of calcein enabled unambiguous identification of tagged *H. midae* larvae from untagged larvae (control treatment) post immersion, and the mark is a visible bright green, fluorescent FITC band on the shell when observed under the fluorescence microscope. Chick (2010a) reported that *H. rubra* juvenile batch-tagged with calcein demonstrated a visible mark in the shell spire regardless of the concentration or immersion period. The results of this study indicate that staining *H. midae* larvae with 50 mg.L⁻¹ calcein results in a brighter epifluorescent mark on the shell than using 25 mg.L⁻¹, regardless of the immersion period. Similar results were obtained by Day et al. (1995), who reported that fluorescent marks in the shell of abalone juveniles, *H. rubra* stained with calcein, were brightest at a concentration of 60 mg.L⁻¹ compared to 10 mg.L⁻¹ irrespective of the immersion time. Furthermore, van der Geest et al. (2011) reported that the percentage of successfully marked bivalve *Loripes lacteus* increased with higher calcein concentrations (100 – 800 mg.L⁻¹).

The most appropriate protocol for quality marking may vary between species (Eads & Layzer 2002). The xylenol orange treatments did not produce a mark on the shell of *H. midae* larvae when reviewed under the FITC filter sets of the fluorescence microscope. These results contradict the claims of Day et al. (1995), who reported that *H. rubra* larvae marked with xylenol orange at concentrations between 20 and 120 mg.L⁻¹ produced a visible mark in the shell after 48 h immersion. The method used by Day et al. (1995) was different from this study in that the *H. rubra* shells were put through a series of water, acetone, and resin baths to facilitate resin penetration. The shell was then embedded in non-fluorescing resin before being viewed under a fluorescence microscope. This may have helped increase the contrast or brightness of the mark, making it easier for a fluorescence microscope to detect it. Other research has used xylenol orange to mark dermal spicules of sea cucumbers (Purcell & Blockmans 2009) and the bone of rabbits (Rahn & Perren 1971), supporting the use of methods that do not implement embedding samples in resin. Day et al. (1995) suggested that 60 mg.L⁻¹ was the best concentration for *H. rubra*, so it is possible that the xylenol orange concentrations used in this study were too low to result in a visible mark in the shell of *H. midae* juveniles. Eads and Layzer (2002) reported that the marking success of batch tags varies between species. The authors showed that calcein (250 mg.L⁻¹) resulted in a distinct marking with rings around the whole shell for bivalves *Actinonaias pectorosa* while the marking was indifferent to that of *Lampsilis cardium*.

The persistence of an epifluorescence mark in the abalone shell is significant for the long-term use of the marking methodology (Eads & Layzer 2002). Consistency in the intensity epifluorescence is important when batch tagging for stock enhancement, as all marked individuals must be quickly identified among wild stock (Fitzpatrick et al. 2013). Calcein-produced epifluorescent marks were clearly visible on tagged *H. midae* juveniles raised under culture conditions after 51 days. Also, there was no difference in the mean brightness of epifluorescence between larvae (before settlement) and juveniles after 51 days post-immersion, suggesting there was no epifluorescence loss over the period. Similar results were obtained by Chick (2010a), who reported that up to 95% of *H. rubra* larval shells were easily distinguishable and that there was no difference in the level of epifluorescence after 260 days. Although this experiment was terminated after 51 days owing to food limitations in the settlement bags, this study suggests that calcein at concentrations of 25 and 50 mg.L⁻¹ are suitable for the successful long-term tagging of *H. midae* juveniles.

In conclusion, it is possible to produce bright and persistent epifluorescence marks using calcein in *H. midae* shells that do not negatively affect the larvae's survival during immersion or at settlement. A 24-hour immersion period to the calcein results in higher larvae survival and is recommended as it subsequently increases the chances of settlement. Although 50 mg.L⁻¹ produced high mean brightness, at 25 mg.L⁻¹, it was still clearly visible and persistent after 51 days, making it easy to identify marked individuals. Calcein is expensive; therefore, if the budget is limited, 25 mg.L⁻¹ is recommended over higher concentrations (50 mg.L⁻¹). These results are of value to studies investigating larval dispersal dynamics and where larvae are released and recaptured, such as stock enhancement or ranching programmes. Further research should refine the optimal calcein concentration to create a persistent mark and determine the long-term effect of calcein staining on the survival and settlement of early-stage juvenile *H. midae* in the natural environment.

CHAPTER 5

Larval seeding of abalone (*Haliotis midae*) in the Eastern Cape, South Africa: A pilot study on the short-term settlement success and its implications for stock enhancement and ranching

5.1 Introduction

The South African abalone, *Haliotis midae*, is a highly prized seafood delicacy in Asian countries, especially Asian metropolitan centres (Sales & Britz 2001, Cook 2016), with China being the largest consumer (Cook 2023). In South Africa, abalone farming began in the early 1990s, and the development and challenges of the industry have been well-documented (Sales & Britz 2001, Troell et al. 2006, Cook 2016, DFFE 2021). The wild abalone stocks along the South African coastline have been reported as heavily depleted (DFFE, 2023) due to increased predation, habitat degradation, poor fisheries management, and overfishing caused by illegal catches (Cook 2014, 2016). In the late 1990s, the growth of the abalone sector led to an interest in stock enhancement as a potential fisheries tool for rehabilitating the depleted wild abalone stock (Godfrey 2003).

The release of hatchery-reared abalone for stock enhancement and ranching has been practised with some success in Japan since the late 1960s (Hamasaki & Kitada 2008). Low recapture rates of seeded juveniles due to predation and high mortality (McCormick et al. 1994, Hansen & Gosselin 2016) combined with the high production cost for rearing juveniles (Olla et al., 1998) negatively affect this practice and have been an ongoing consideration. These have led to an interest in seeding with abalone larvae, which could provide significant benefits for abalone restoration. The advantages of seeding abalone larvae include cost savings, as using larvae would reduce rearing time in the hatchery from nine months to as little as seven days (Mills-Orcutt et al. 2020), freeing up space to raise another batch of larvae. Secondly, many abalone larvae can be produced in a basic hatchery (Preece et al. 2000), which could be built at a fraction of the cost of an on-growing farm. Thirdly, the short period of rearing larvae may

uncouple abalone farms from the high electricity and labour costs since abalone will grow at minimal cost to the farmer (Cook 2016).

Seeding of abalone larvae has not been tested as extensively as seeding of juvenile abalone and is rarely documented. This restoration tool so far has been attempted on an experimental scale in New Zealand (Tong et al. 1987), in Australia (Preece et al. 2000, Heasman et al. 2004, Chick 2010a), and in the United States of America (Mills-Orcutt et al. 2020). Heasman et al. (2004), Chick (2010a), Mills-Orcutt et al. (2020) used permeable tents to cover their settlement places when releasing larvae to improve the initial settlement. Heasman et al. (2004), Chick (2010a), Mills-Orcutt et al. (2020) also used chemical inducers such as Gamma-aminobutyric acid (GABA) as one of the factors to ensure the successful settlement of released larvae.

While several studies have been conducted on abalone larval seeding (Tong et al. 1987, Preece et al. 2000, Heasman et al. 2004, Chick 2010a, Mills-Orcutt et al. 2020), there is no information on its success in South Africa. Studies on abalone larval seeding in other countries have typically been conducted in calm seas that are not exposed to intense or high-energy wave action. However, the Eastern Cape coastline of South Africa is a highly exposed, high-energy environment, with an annual wave height of over 3 meters (Goschen et al. 2012). Upwelling and downwelling events also frequently occur along the coastline (Goschen et al. 2012). This suggests that a tent-like structure is unlikely to work under South African conditions. McCarroll et al. (2021) suggested that a zone of shallow gullies and bays under lower-energy wave conditions is possibly the optimal release point to maximise residency times within the preferred rocky, shallow subtidal habitat of *H. midae*.

This study aimed to investigate the viability of seeding *H. midae* larvae as a tool for abalone ranching and stock enhancement programme on the Eastern Cape coastline of South Africa. To test a technique to seed competent abalone larvae, a pilot study was designed to determine the settlement, short-term survival and growth of released larvae into shallow inshore gully and bay of low energy wave regime by quantifying the number of recovered abalone from seeding. It was hypothesised that the number of seeded abalone larvae would decline rapidly within the first week, affecting the overall survival. This study is essential as it will provide important information on potential parameters governing the limitations and successes of a reseeding initiative, leading to the development of seeding trials at a commercially realistic scale.

5.2 Materials and methods

Abalone larval rearing

Abalone larvae were spawned and raised in the commercially operating hatchery at Wild Coast Abalone Farm (Pty) Ltd (32°45'05" S 28°16'27" E), South Africa. Larvae were raised in a flow-through rearing system in a controlled temperature room maintained at 18 °C and photoperiod of 12h:12h. Larvae developed until they were competent to settle; this coincided with five days (Mzozo et al. 2021).

Experimental design

i. *Study site*

A suitable site for abalone larval settlement and juvenile growth was identified using criteria proposed by McCarroll et al. (2021), who suggested that the ideal inshore intertidal region release point is from the gullies and small bays just west of the cape. The abalone seeding sites were located at Noordhoek, adjacent to Cape Recife (West side) on the coastline of the metropolitan area of Qheberha, Eastern Cape, South Africa (Fig. 1). The sites (East, Middle, and West) were selected within the ranching concession area EC1 at the Noordhoek Ski Boat Club. Seeding sites are located in a gully and bay environment in a very shallow inshore intertidal zone, with relatively calm conditions beyond the reach of the surge during low tide. After selection, the sites were marked with Global Positioning System (GPS) coordinates at each site. The three sites are subtidal, with a 1 ± 0.2 m CD water depth. The sea floor comprises of inshore broken reef and boulder bed covered in predominately agal turf with crustose coralline algae and invertebrates (sea urchins, starfish, limpets), which create crevices for protection, encrusted algae both for inducing settlement and good food availability (Moriarty 2019).

Plastic sheets (30 x 10 cm) made of high-rigidity PVC material were placed in a water channel with the out-flow natural seawater from the settlement tanks in the abalone hatchery and allowed the natural growth of a 1mm thick layer of crustose coralline algae over four months. Six crustose coralline algae-encrusted settlement plates were attached to steel weights, and two were placed at each site.

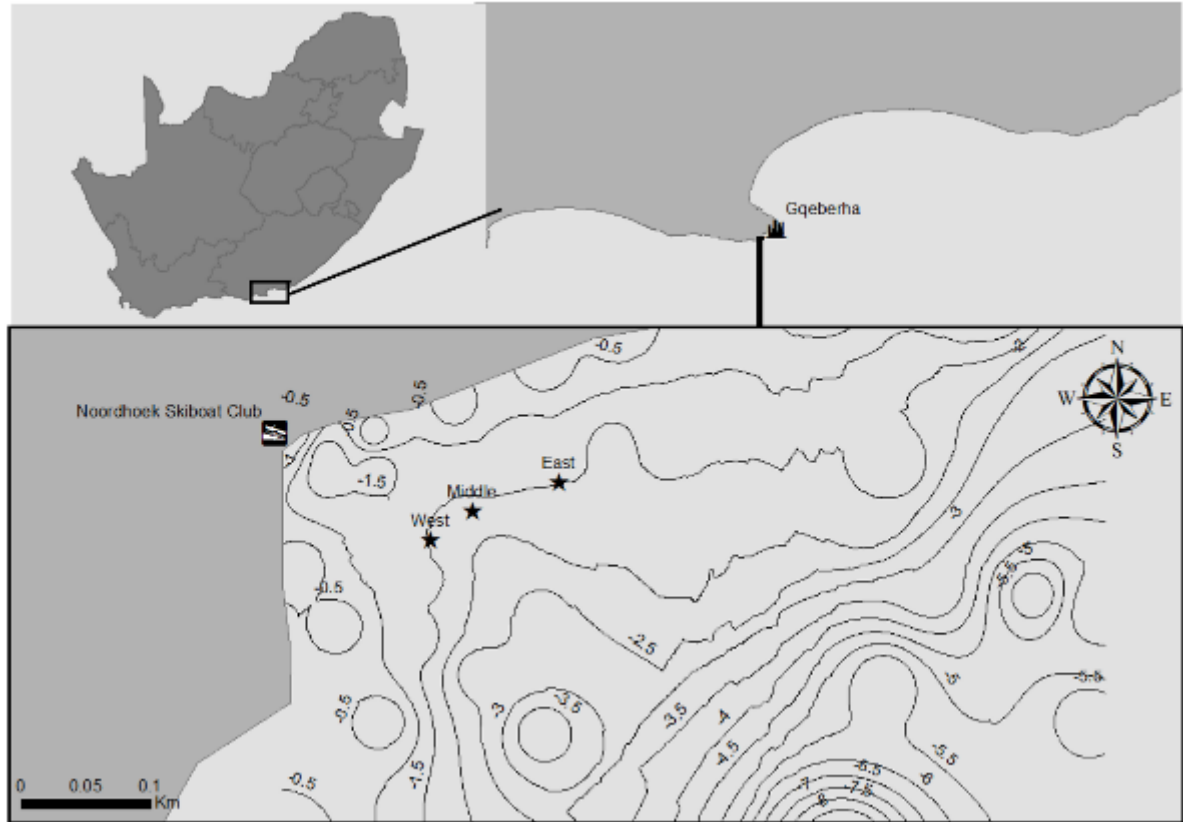


Figure 5.1 Site Map showing three seeding sites (West, Middle and East) in Noordhoek, Eastern Cape

ii. *Staining method*

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Before seeding, five-day-old competent *H. midae* larvae (70 larvae. mL⁻¹) were stained by immersing in a 25 L aquarium filled with seawater (1 µm filtered and UV treated) for 24 hours and tagged using 25 mg.L⁻¹ calcein (206700, Sigma–Aldrich). After immersion, larvae were rinsed by gently pouring them onto an 80 µm mesh screen to remove excess dye before being gently rinsed with clean seawater.

iii. *Tag confirmation*

Tag confirmation treatment was conducted at the Wild Coast Abalone farm in the hatchery larval culture room using diatom-coated settlement bags to confirm that the stained abalone larvae could settle post-staining, and that batch-tagging was successful. The six settlement bags were coated on the inside surface with natural diatoms from incoming seawater. Approximately 20,000 stained larvae were introduced into each bag and allowed to settle on the diatom layer. During the first four days of settlement, the water flow was switched off to avoid flushing the swimming larvae out of the bags. Each bag was aerated, and the temperature was maintained

at 18 ± 0.5 °C. Larvae were raised in the settlement bags for five weeks, after which the juveniles were debagged, and the stained shell was confirmed under the fluorescence microscope (Olympus BX-61).

iv. *Transport and release of larval abalone*

Into each of the thirty 25 L buckets, approximately 500,000 larvae were added, and the top 2-3 L of air was replaced with oxygen. The buckets were immediately transported from Wild Coast Abalone Farm (Pty) Ltd, East London, to the study site at Noordhoek, Gqeberha, South Africa, a journey of approximately five hours. The transportation method has been previously assessed, and the results indicated high survival and settlement potential under controlled conditions (Bajaba, 2021). Abalone larvae were slowly acclimated to onsite seawater temperature over 15 minutes and then released at the three sites ($\pm 5,000,000$ larvae per site). Mzozo et al. (2023) reported that the settlement of *H. midae* increases with increasing concentrations of KCl, and settlement decreases when the KCl concentration was reduced due to flushing. This suggests that KCl might be ineffective when releasing abalone larvae into the ocean owing to flushing.

v. *Sampling of abalone*

Plankton samples were collected 15 and 20 hours after releasing larvae. An 80 µm net was attached to a 12 VDC submersible (1591 pm) pump, and water was pumped for 5 min below the surface. Three samples were collected from each site, and the net was carefully rinsed into separate sampling jars. A settlement plate from each site was removed by divers after 20 hr and brought up to the boat, where it was carefully wiped with a sponge and rinsed to collect abalone larvae that might have settled on the plate, which was immediately frozen to kill all organisms. The settlement plates were returned to the reef. Samples were analysed under a dissecting microscope (Best Scope–3040T, Lasec South Africa)

The three seeding sites were sampled for juvenile abalone by divers three times post-larval release (t0), 314 days after release (t1), 442 days after release (t2), and 608 days after release (t3). During the first sampling of juvenile abalone (t1), both settlement plates were removed and checked for abalone at each site. Unfortunately, most settlement plates had been destroyed and were not replaced. Abalone quadrant surveys entailed divers counting all cryptic and emergent juvenile abalone within a 30 x 30 cm quadrant placed on the seabed for 10 minutes per quadrant. Divers only focused on areas with suitable habitat conditions for juvenile abalone, particularly in crevasses in broken reefs and under boulders covered with crustose coralline

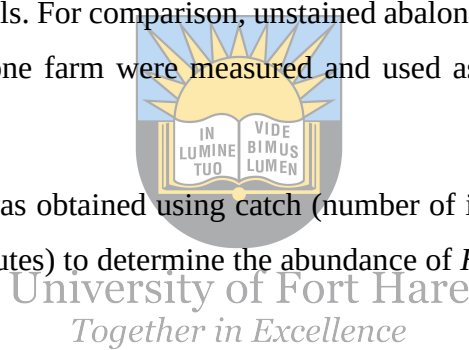
algae and sea urchins. Once the divers had completed a quadrant, they moved more than 30 cm but less than one meter away from the previous quadrant while remaining in suitable abalone habitat. Each diver searched for an hour using a focused spiral sampling technique, with six quadrats being sampled per site per sampling event (t1, t2, t3). The surface area covered during sampling was used to calculate wild and seeded abalone population density.

Divers recorded the shell length of all juvenile abalone found within the quadrant; all abalone smaller than 50 mm shell length were retained and placed in 70% ethanol immediately after the dive. The data collected for abalone shell length were used to calculate the average growth rate per day in terms of shell length as follows:

Growth rate (mm.day^{-1}) = shell length (mm) /no. of days in the ocean

Any biofouling on the spire of a shell was carefully scrapped off. Abalone shells were observed under a fluorescence microscope (Olympus BX-61) using the FITC set (green fluorescent) to identify calcein-marked shells. For comparison, unstained abalone shells from a similar cohort reared at Wild Coast Abalone farm were measured and used as a control against the wild-caught individuals.

Catch per sampling event was obtained using catch (number of individual abalone) per diver and effort (dive time in minutes) to determine the abundance of *H. midae* release on the study sites.



Statistical analysis

Data variables were checked to see if they met parametric test assumptions using the Shapiro–Wilk test for normality and Levene's test for homogeneity. The data was analysed using Welch's analysis of variance (ANOVA), which is robust to non-equal variance among treatments to compare variation Catch Per Unit Effort (CPUE). The CPUE between divers was standardised. The Kruskal-Wallis Test was used to investigate the differences in growth and population density, as Levene's test revealed a non-homogeneity of variances. When significant differences ($p < 0.05$) were found, Tukey's posthoc and pairwise comparison tests were used to determine specific differences among treatments. IBM SPSS (version 26) was used for all data analyses, with all data presented as mean $\pm$ SE.

5.3 Results

Tag-confirmation

Abalone in the tag confirmation treatment settled well post-staining with 25 mg. L⁻¹ calcein with an average density of 8.72 ± 1.14 larvae.cm². Also all juvenile abalone were positively marked with the calcein tag.

Settlement of hatchery-reared larvae

Sixteen hours post-release, no abalone larvae were found swimming in the water column across all three release sites at Noordhoek. No abalone larvae were attached to the settlement plates from each release site one-day post-release.

Population density

Quadrant surveys were conducted three times over a year-and-a-half period for all sites. Due to poor diving conditions, the data point from t1 (314 days), with only one site completed, was excluded from the analysis that compared between sites. The abundance is reported in terms of the density of seed abalone and wild abalone and the proportion of seed abalone in the sampled population. The abundance of seed and wild *H. midae* varied between the three sites. On the West site, 6.3% of all recaptured abalone in all the sampling events were seeded abalone (2.59 seeded.m² vs 44.90 wild.m²). On the middle site, there was high natural recruitment of juveniles but seeded abalone still contributed 3.5% of all recaptured abalone. At the same time, the East site had the lowest recruitment and seeded recapture (Table 5.1). The density of recaptured seeded juveniles was not different between sampling events.

Table 5.1 Summary of released sites surveyed at Noordhoek (Eastern Cape), including total seed of recaptured abalone, total seed recapture, mean proportion of seed abalone and mean density of wild and seed *Haliotis midae* after 608 days at liberty.

Site	Total area sampled (m ²)	Total number of recaptured abalone	Proportion of recaptured seed abalone (%)	Density of wild abalone (per m ²)	Density of seeded abalone (per m ²)
West	2.43	111	6.31%	44.90 ± 10.40^a	2.59 ± 1.11^a
Middle	2.16	113	3.54%	50.46 ± 7.42^a	1.85 ± 1.07^a
East	1.35	28	0	18.51 ± 4.54^b	0 ^a

Different letters in the same column indicate significant differences $p < 0.05$

Growth rate

The growth rate of recaptured seed *H. midae* juvenile abalone was variable through time ($F(2)=6.55$, $p = 0.010$). The growth rate was not significantly different between the three release sites. A fully developed veliger was released, and after 14 months (442 days) at liberty, the mean shell length of the recaptured seed juveniles was 11.33 ± 1.18 mm, giving an average daily growth rate of $0.026 \text{ mm. day}^{-1}$. After nearly 20 months (608 days) at liberty, recaptured seed juveniles grew at an average of $0.037 \text{ mm. day}^{-1}$ (Table. 5.2). Knowledge of growth rate from the study by Genade et al. (1988) was used to compare the growth rate of recaptured seed *H. midae* juveniles.

Table 5.2 Comparing growth of hatchery released *Haliotis midae* juveniles collected from the wild and a those from a controlled laboratory experiment.

Wild-reared juveniles (this study)		Experimental reared juvenile (Genade et al. 1988)	
Age and shell length (mm)	Daily growth rate (mm.day ⁻¹)	Age and shell length (mm)	Daily growth rate (mm.day ⁻¹)
442 days: 11.33 ± 0.09	0.026 ± 0.002	12 months: 11.8	0.032
608 days: 22.25 ± 1.030	0.037 ± 0.001	24 months: 34.4	0.047

The distribution of shell length of all recaptured juveniles of the 20-month periods across all sites showed growth of abalone larvae on the reef. After 442 days, shell sizes that ranged between 5-10 mm had high modal sizes. Then, after 608 days, shell sizes that ranged between 10 -20 mm high modal size were observed (Fig. 5.2).

Abalone abundance on released site

The catch rate of *H. midae* decreased after the second sampling period, and the abundance was down by 11.54% after day 608 at liberty (t_3). A significant difference in CPUE was observed between sites ($F(2)= 5.98$, $p = 0.038$). There was no significant difference between the seeded juveniles recovered from the West and Middle sites. The CPUE between sites showed very low catches in the East site ($10 \text{ abalone.h}^{-1}$) compared to the West site, where catches attained were almost three times high ($27 \text{ abalone.h}^{-1}$).

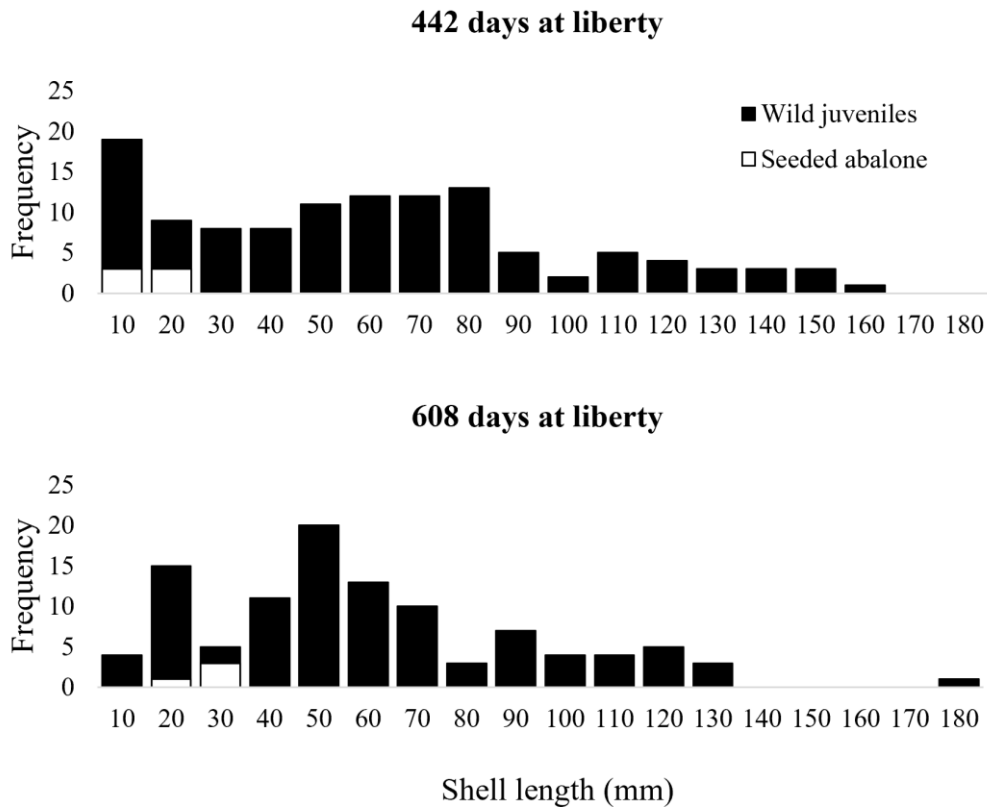


Figure 5.2 Size range length-frequency distribution of recapture (wild and released) juvenile *Haliotis midae* after 408 and 608 days at liberty.

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5.4 Discussion

During the veliger stage, abalone larvae are free swimming and could potentially disperse for kilometres from their source before settlement (McCarroll et al. 2021). Prince et al. (1987) stated that the dispersal abilities of haliotid larvae are limited only by the time available before settlement. In this study, *H. midae* larvae were competent to settle, reducing their dispersal window period to less than 24 hours. Competent *H. midae* larvae were seeded in the inshore gully environment at Noordhoek during high tide. The physical forces at the time of larval seeding were possibly too powerful and abalone larvae might have been passively transported by the current beyond the study site. McCarroll et al. (2021) indicated that larvae released in the protected bay and gully areas near the shoreline are more likely to stay within the area and not disperse far off. Shephard et al. (1992) reported that bays generate eddy systems with countercurrents and stagnation zones in the presence of currents, concentrating plankton to the background levels. Alldredge and Hammer (1980) reported the downward vertical component

from the reverse current, which assists in concentrating larvae at the surface and the flood current dispersed larvae inshore. This movement suggests that there might be chances that release larvae might settle in the gully and bay environment.

Settlement of abalone larvae on the natural reef is crucial for the success of abalone larval seeding (Heasman et al. 2004). After 20 hours post-release in this study, no larvae were found swimming in the water column at all three sites. Abalone larvae (*H. midae*) were competent to settle at the time of release. Older larvae settle faster and spend less time searching and exploring the substrate when in the presence of a suitable substrate (Tong et al. 1987). Moss and Tong (1992b) observed that older *H. iris* larvae settle quicker (90% after 2 hours post seeding) than younger larvae (66% after 2 hours post seeding) in the presence of suitable substrate. The abalone larvae might have settled after 20 h post-seeding. Unfortunately, the attempt in this study to quantify the settlement success of *H. midae* larvae using settlement plates encrusted with crustose coralline algae was unsuccessful; no post-larvae were identified on the settlement plates. This could be attributed to (i) the likelihood that larvae might have considered other natural settlement substrates available adjacent to the settlement plate. Studies by Roberts and Lapworth (2001), Huggett et al. (2005), Chick (2010a) reported that rocks encrusted with crustose coralline algae increase abalone larval settlement as they produce chemical cues (phycobiliproteins), which abalone larvae sense on the coralline algae's surface and settle. This study's seeding site consists of broken reefs and boulders encrusted with coralline algae and sea urchins. Therefore, larvae likely might have settled on the boulders instead of artificial settlement plates. (ii) the physical forces during larval seeding were too powerful, possibly causing dispersal beyond the settlement plates. Boxshall (2000) reported that when larvae crawl and explore the surface using their foot, if the water-free-stream velocity over the substrate surface is too high, it can passively lift the larvae into the water column, possibly allowing them to land and settle elsewhere. (iii) the diver's handling of the settlement places with newly settled larvae might have caused possible loss of abalone larvae in the transfer between the reef and the boat. Unfortunately, the results could not be used to estimate the initial settlement of *H. midae* larvae released on the sites, and future studies should address this.

Survival of hatchery-reared released abalone in the wild is vital for the success of abalone seeding as a means to improve depleted wild stocks (Hansen & Gosselin 2016). Previous studies have reported low survival for seeded larvae (less than 1%), which is near total mortality of seeded larvae (Preece et al. 1997, Heasman et al. 2004, Chick 2010a, Mills-Orcutt et al.

2020). While several variables affect seed survival, site characteristics are crucial (Gerrity & Schiel 2024). This study shows that the survival of recaptured seed juvenile abalone was not different between the West and the Middle site, and no abalone was recaptured on the East site. Although the population density of seeded larvae was less than that of wild abalone, a similar trend was observed in the density of wild juvenile abalone across the three sites. The density of wild *H. midae* juveniles was lower on the East site than the two other sites, with the West site exhibiting the highest density, followed by the Middle site. Wild abalone's presence in higher density indicates better conditions at the sites (Witte 2017, Gerrity & Schiel 2024). Therefore, this suggests that the West and Middle sites had better conditions than the East site; hence, higher survivorship of *H. midae* juveniles was achieved on sites, and no recaptured seed juveniles were found on the East site with low wild density. Secondly, the West and middle sites are in shallow bay-like areas, which are protected zones. McCarrol et al. (2021) reported that seeding occurring under lower-energy wave conditions is predicted to increase residence in the protected zone. The East site is in the mouth of a gully and is subjected to more surges during high tide. The conditions on the East site may be too rough for abalone larval settlement, which could possibly result in poor natural recruitment for seeded larval flushed pass the mouth of the gully by the Eastern longshore current. It is important to note that divers focused their sampling effort on areas they consider favourable to support juvenile abalone. This would have introduced a selective bias and may have overestimated the abundance of juveniles in the area. This estimate should, therefore, be considered with caution.

The growth rate of abalone in the wild can be influenced by microhabitat differences, food supply and quality differences, seasonal variations in temperature, and water movement (Heasman et al. 2004). The growth rate was no different between the West and Middle sites. The West and Middle sites were close, which suggests that environmental conditions were similar. Godfrey (2003) found high variation in growth rates at Cape Recife sites at least two kilometres apart, whereas the three sites in this study were within 100 meters apart.

After nine months, juvenile abalone from the same batch cohort of seeded abalone could no longer be tracked on the farm. The effect of environmental factors on the growth rate of released larval *H. midae* was comparable to the laboratory growth rate reported by Genade et al. (1988). Genade et al. (1988) raised *H. midae* juveniles under a wide range (10 -27 °C) of ambient temperatures and unfiltered seawater. Larvae were fed natural diatoms, and juveniles changed to fed pieces of *Plocamium* sp. and *Ulva fasciata*, similar to the conditions that *H. midae* were exposed to in the wild. The growth rate of recaptured seed *H. midae* was similar to

the growth rate of hatchery-raised juveniles. Genade et al. (1988) recorded 0.032 mm. day⁻¹ growth for *H. midae* juveniles after 12 months under controlled conditions (see Table 6.2). These rates align with the estimated growth rate reported in this study. This confirms that growth rates of seeded abalone were not static, which is a significant result for the success of abalone ranching and stock enhancement.

Stock abundance is an important parameter in fisheries management (Hart & Gorfine 1997). A spatial difference between the three sites is evident, with the highest *H. midae* abundance on the West site. A temporal difference in abundance is also evident, with significantly lower CPUE after 608 days at liberty compared to 442 days at liberty. The CPUE was 11.5% less than the second sampling event. The most likely explanation for the decrease is that juveniles might have emigrated beyond the sampling sites. Abalone are known to utilise different habitats in their life cycle (Chick et al. 2013). In this study, the variation of size frequency distribution among two sampling times indicates a higher proportion of smaller abalone in the shallow intertidal zone, and the proportion of abalone with a size range over 70 is significantly reduced in the sampling site. This suggests that bigger abalone might have moved offshore in search of crevice space, contributing to a substantial decline in the recapture of abalone after release. A similar trend was reported by Witte (2018), with a high proportion of small size frequencies found in shallow areas in Cape Recife. Glazer (2005) reported that the model of emigration assumes that landscape features must not affect the movements of the population. In this study, the sampling sites are located in a gully open to the offshore, suggesting that abalone emigration might have been possible. Witte (2017) reported that South African abalone (*H. midae*) recruit mainly in the inshore sub-tidal zone and moves offshore as they grow.

In conclusion, this pilot study demonstrated that seeding abalone larvae *H. midae* along the coastline of the Eastern Cape, South Africa, is a potentially viable ranching method. While the recapture of seeded abalone was low, it does not necessarily indicate poor survival, as it may be a consequence of a limited sampling protocol. Once larval seeding protocols are optimised, the seeding of abalone larvae could be a valuable tool for stock enhancement programmes and abalone ranching. Future studies should consider increasing the number of artificial settlement plates and providing a settlement area for abalone larvae, which could be used to monitor initial settlement. Conducting more regular sampling (i.e. two times a month for the first month and three times a year) and using different sampling methods (i.e. focus on surveying the full range of habitat to be able to predict the juvenile population density more accurately) would also improve our understanding of recruitment. Future studies should also consider investigating

release points based on wave direction, water depth, and tidal cycle, as optimising these parameters would ensure larvae remain in the target area where they can settle and establish themselves.



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CHAPTER 6

General discussion

The decline of the South African abalone fishery over the past four decades has been well documented (Raemaekers & Britz 2009, Cook 2023, DFFE 2023). Unfortunately, the South African government's fishery management strategies have failed to improve the situation, and recent reports suggest that the wild stock is heavily depleted, facing significant illegal fishing pressure (DFFE 2023). As outlined in Chapter 1, abalone ranching and stock enhancement have been proposed as methods to rehabilitate wild abalone populations in South Africa. Currently, juvenile abalone are utilized for ranching; however, the costs associated with rearing juvenile and the high mortality rates in the wild have led to increased interest in seeding abalone larvae instead.

Abalone larval ranching of *Haliotis midae* has been considered a potential tool for rehabilitating fisheries in depleted areas, as it could reduce the costs associated with hatchery rearing. One major concern with this technique is the inconsistent settlement of larvae, which is a common issue in abalone hatcheries. Inconsistent settlement could result in extended settlement windows and potential larval dispersal outside the designated seeding area. While larval dispersal could benefit stock enhancement, ranching is subject to specific regulations; if the stock moves out of its designated area, the right holder loses the ability to retrieve it (South African Government Gazette 2010). Additionally, there is currently no information regarding the seeding of *H. midae* larvae along the South African coastline.

It is crucial to develop the necessary techniques for the economically viable release of *H. midae* larvae along the South African coast. The research described in this thesis has provided baseline information for implementing abalone larval ranching to enhance wild abalone stocks in South Africa. The results presented in this thesis serve as a foundational method for establishing abalone ranching and improving larval settlement in abalone hatcheries.

The effect of temperature on the rate of development and settlement of the *H. midae* larvae

Increased temperatures within the optimal range for *H. midae* may enhance the metabolic rate sufficiently to support increased growth (Pedroso 2017). The results in Chapter 2 confirmed that temperature affects the development and settlement of *H. midae* larvae, with a higher growth rate observed in larvae raised at warmer temperatures. This research includes the development of a degree-day model to predict growth and the timing of initial settlement for *H. midae* larvae across various temperatures.

Settlement and metamorphosis are critical in developing an abalone fishery, as they dictate the success or failure of larval seeding. Poor settlement caused by larvae dispersing outside the seeding area can considerably impact abalone ranching and stock enhancement opportunities. Narrowing the settlement window period could reduce larval dispersal, thereby increasing the likelihood of settlement within the seeding area. To achieve this, it's essential to understand the development timeline of abalone larvae and their settlement timing across different temperatures. Utilizing a degree-day model can facilitate this understanding. The model can predict the developmental stages for *H. midae* larvae across a range of temperatures, which is vital for effective planning of the seeding process (i.e., synchronizing the degree-day timing when larvae are competent to settle with low tide at the release site) and managing other logistics.

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Additionally, modeling the degree days of *H. midae* larval development could assist abalone farms in controlling production schedules effectively, impacting spawning, diatom availability, nursery space, and other logistical aspects.

A strong relationship between temperature and the settlement of *H. midae* larvae was observed; more larvae settled at warmer temperatures (22°C) compared to lower temperatures (14°C). The temperature range of 18°C to 22°C aligns with the sea temperatures experienced by *H. midae* in their natural habitat. This finding suggests that releasing larvae at warmer temperatures might narrow the settlement window, thereby reducing the likelihood of larvae dispersing outside the seeding area.

Investigating the effect of chemical and biological settlement cues on *H. midae* larval settlement in a flow-through system

For abalone larval ranching and stock enhancement, competent abalone larvae are released just before settlement to boost the likelihood of successful settlement on the surrounding reef. Larval settlement is influenced by chemical cues, which indicate a suitable environment for newly recruited individuals (Huggett et al. 2005). The effect of potassium chloride (KCl) as a settlement inducer has been widely recognized across various marine invertebrates (Yool et al. 1986, Bryan & Qian 1998, Gapasin & Polohan 2004, Kang et al. 2004, Li et al. 2006, Yu et al. 2010). However, most research has focused on improving settlement under controlled conditions, typically in stagnant water, resulting in limited information on whether these techniques can be applied effectively for ocean seeding.

In this study, abalone larvae (*H. midae*) were exposed to settlement inducers (both chemical and biological cues) in a flow-through system that simulates natural conditions. Based on findings from Chapter 3, it was determined that settlement inducers are ineffective in a flow-through system, suggesting this method is not viable for ocean seeding. Barlow (1990) reported that gamma-aminobutyric acid (GABA) does not paralyze cilia at low concentrations and does not induce larval settlement. This implies that exposing *H. midae* larvae to 10 mM KCl before release may not enhance settlement in the ocean, as the concentration may become diluted, leading larvae to swim off the substrate and disperse.

Nonetheless, the technique of exposing *H. midae* larvae to a combination of 10 mM KCl and free-swimming *Nitzschia* could be beneficial in hatcheries, where larvae are continuously exposed to the inducer until metamorphosis. Therefore, while exposing *H. midae* larvae to 10 mM KCl and *Nitzschia* before ocean release is not recommended, as effective settlement cues are only viable at high concentrations, this approach could have applications in hatchery environments.

Determine the optimal dose of staining fluorescent dye for shell marking and its effects larval survival and settlement

Measuring the survival of hatchery-reared and released abalone is essential for assessing the success of stock enhancement programs (Chick 2010). Accurately distinguishing hatchery-reared from wild abalone is crucial for evaluating the survival of released larvae. Abalone larvae respond differently to chemical exposures, so a suitable concentration of fluorescent dye

should be determined that marks the shell without negatively impacting the settlement and survival of *H. midae* larvae. Data from Chapter 4 indicated that *H. midae* larvae were successfully batch-tagged using calcein. This method allowed for individual identification via a fluorescence microscope, although not via the naked eye.

The results also demonstrated that calcein positively affects larval survival, with higher survival rates observed in calcein-treated groups compared to control groups. This unexpected finding suggests that calcein may have an antibiotic or antiviral effect on the water's microbial community. Although this study did not evaluate microorganism concentration, pausing the flow of UV-treated water during the staining process might have allowed for increased microbial growth, which could have influenced the larvae in the control treatment.

The immersion period also impacts the survival and settlement of *H. midae* larvae post-batch tagging. A 24-hour immersion period showed higher survival rates, while a 48-hour immersion period resulted in higher initial settlement rates. Based on the findings from Chapter 4, a 24-hour immersion period is recommended for marking *H. midae* larvae. However, rapid settlement or a reduced settlement window is advantageous for abalone ranching and stock enhancement programs, as it minimizes the risk of larval dispersal outside the designated seeding area.

The selection of fluorescent dye for batch-tagging abalone larvae should not solely depend on tag brightness; post-staining survival also plays a crucial role. Future studies aim to assess staining abalone larvae at a later development stage to encourage immediate settlement following a 24-hour immersion period.

The epifluorescence mark of calcein was successfully embedded in the spiral of the growing shell of *H. midae* abalone larvae. In contrast, marking with xylenol orange was unsuccessful. Although xylenol orange at 50 mg.L⁻¹ produced high brightness, calcein demonstrated clear visibility and persistence on the shell at 25 mg.L⁻¹ after 51 days, facilitating easy identification of marked individuals. The persistence of the tag was only measured for 51 days due to food limitations. However, studies by Day et al. (1995) and Chick (2010) suggest that the calcein tag can last for up to two years on an abalone shell, indicating the potential for longer-lasting tags on *H. midae* juveniles.

Calcein is expensive; therefore, a combination of a 24-hour immersion and 25 mg.L⁻¹ calcein concentration is recommended to produce bright and persistent epifluorescence marks without negatively affecting larvae survival and the post-immersion settlement percentage.

Assessing the settlement success and short-term survival of seeding *H. midae* larvae into the ocean

There is limited information on the seeding of *H. midae* larvae in South Africa. One of the main concerns with this technique is the dispersal of larvae outside the seeding area and the subsequent survival of the released juveniles. The regulations governing abalone ranching prohibit rights holders from retrieving any stock that moves beyond their designated ranching area (South African Government Gazette, 2010). Recommendations from previous chapters that explored methods to improve abalone larval settlement in the wild were applied in the release of a large number of abalone larvae into the ocean. This aimed to investigate the potential for enhancing local abalone populations through larval seeding.

The results presented in Chapter 5 provide innovative insights, demonstrating that a significant number of *H. midae* larvae can be successfully released to the natural reefs along the South African coastline. This method represents a cost-effective and rapid solution for seeding trials, where millions of larvae can be produced in a basic hatchery within a week.

The experiments discussed in Chapter 5 yielded encouraging results regarding the survival of seeded abalone larvae on the natural reef. The released larvae successfully settled and survived, with a notable number of juveniles recaptured. Although the density of seeded abalone was low, this does not necessarily indicate poor survival, as it may result from a limited sampling protocol. It is essential to note that divers focused their sampling in areas they believed were most suitable for juvenile abalone, which may introduce a selective bias and overestimate the abundance of juveniles on the reef. Future studies should aim to survey a broader range of suitable habitats on the reef to more accurately assess juvenile population density.

The reef structure at the release site (Noordhoek, Gqeberha, Eastern Cape) was reported to feature boulders, holes, or crevices with low sand cover (Witte, 2017), providing ideal conditions for abalone larval settlement. This suggests that some larvae likely remained on or near the Noordhoek reef. The results of this study emphasize the suitability of the selected seeding site; released larvae not only survived, but the growth rate of the juvenile abalone did not appear to be stunted.

Challenges were faced in quantifying the settlement success of *H. midae* larvae using settlement plates encrusted with crustose coralline algae, which proved ineffective. Future studies should focus on developing a reliable method to quantify initial settlement on the South African coastline.

Overall, the results demonstrate that seeding *H. midae* larvae along the coastline of the Eastern Cape, South Africa, is a potentially viable ranching and stock enhancement method.

Conclusion

This study has enhanced our understanding of the settlement characteristics of *H. midae* larvae. Warmer temperatures (18 – 22 °C) increase the developmental rate of *H. midae* larvae, with each 2-degree rise resulting in higher settlement rates. This increased settlement narrows the settlement window and reduces dispersal. Additionally, the study indicates that using chemical cues prior to seeding may not enhance post-release settlement, as chemical cues are only effective when concentrations can be maintained at higher levels. A combination of a 24-hour immersion and a concentration of 25 mg.L⁻¹ calcein is recommended to produce bright and persistent epifluorescence marks without negatively impacting larval survival.

This research provides a baseline methodology for the release of *H. midae* larvae along the South African coastline for abalone ranching and stock enhancement. With further improvements in the seeding technique, larval seeding could become a valuable tool for stock enhancement programs and abalone ranching.

Future studies

As mentioned, there is currently a lack of research on using *H. midae* larvae for stock enhancement and ranching opportunities. Therefore, studies that complement and build on the findings of this thesis will aid in evaluating both the ecological and financial viability of seeding abalone larvae.

Further investigation into the long-term effects of temperature on the development and growth of recently settled juvenile abalone would be beneficial. Understanding how variations in water temperature influence the settlement of *H. midae* larvae is crucial, as raising and releasing abalone larvae at different temperatures may yield differing settlement success rates. Such research could provide essential insights into the viability of year-round larval seeding without increasing dispersal risks and potential mortality.

Future studies could also consider the use of artificial settlement plates to enhance the potential settlement area for abalone larvae, thereby improving overall success rates.

CHAPTER 7

References

- Abagold. 2021. Abagold integrated annual report. *Report*:1–116.
- Abdel-Rouf, N. 2012. Microalgae and wastewater treatment. *Saudi Journal of Biological Sciences* 19:257–275. <https://doi.org/10.1016/j.sjbs.2012.04.005>
- Alfaro, A., Young, T. & Bowden, K. 2014. Neurophysiological control of swimming behaviour, attachment and metamorphosis in black-footed abalone (*Haliotis iris*) larvae. *New Zealand Journal of Marine and Freshwater Research* 48:314–334. <https://doi.org/10.1080/00288330.2014.918886>
- Al-Rashdi, K. & Iwao, T. 2008. Abalone, *Haliotis mariae* (Wood 1828), hatchery and seed production trials in Oman. *Journal of Agricultural and Marine Science* 13:53-63
- Alldredge, A. L. & Hamner, W. H. 1980. Recurring aggregation of zooplankton by a tidal current. *Estuarine and Coastal Marine Science* 10: 31–37
- Baloun, A. J. & Morse, D. E. 1984. Ionic control of settlement and metamorphosis in Larval *Haliotis Rufescens* (Gastropoda). *Biological Bulletin* 167:124–138.
- Bajaba, S. 2022. South African abalone (*Haliotis midae*) larval rearing and transportation at Wild Coast Abalone (Pty) Ltd, Haga Haga, Eastern Cape, South Africa. MSc thesis, University of Fort Hare, 1-49.
- Barlow, L. A. 1990. Electrophysiological and behavioral responses of larvae of the red abalone (*Haliotis rufescens*) to settlement-inducing substances. *Bulletin of Marine Science* 46:537–554.
- Bates, D. Maechler, M., Bolker, B., Walker, S. 2015. Fitting linear mixed-effects models using lme4. *Journal of Statistical Software* 67(1):1–48.

- Bell, J. D., Leber, K. M., Blankenship, H. L., Loneragan, N. R. & Masuda, R. 2008. A new era for restocking, stock enhancement and sea ranching of coastal fisheries resources. *Reviews in Fisheries Science* 16:1–9. <https://doi.org/10.1080/10641260701776951>
- Boxshall, A. J. 2000. The importance of flow and settlement cues to larvae of the abalone, *Haliotis rufescens* Swainson. *Journal of Experimental Marine Biology and Ecology* 254:143–167. [https://doi.org/10.1016/S0022-0981\(00\)00274-4](https://doi.org/10.1016/S0022-0981(00)00274-4)
- Britz, P.J., Lee, B., Botes, L. 2009. AISA 2009 Aquaculture benchmarking survey: primary production and markets. *AISA report produced by Enviro-fish Africa* (Pty) Ltd, 1- 117.
- Britz, P.J., Hecht, T., Mangold, S. 1997. Effect of temperature on growth, feed consumption and nutritional indices of *Haliotis midae* fed a formulated diet. *Aquaculture* 152: 191–203.
- Bryan, P. J. & Qian, P. 1998. Induction of larval attachment and metamorphosis in the abalone *Haliotis diversicolor* (Reeve). *Journal of Experimental Marine Biology and Ecology* 223:39–51. [https://doi.org/10.1016/S0022-0981\(97\)00156-1](https://doi.org/10.1016/S0022-0981(97)00156-1)
- Chezik, K. A., Lester, N. P. & Venturelli, P. A. 2014. Fish growth and degree-days II: Selecting a base temperature for an among-population study. *Canadian Journal of Fisheries and Aquatic Sciences* 71:1303–1311. <https://doi.org/10.1139/cjfas-2013-0615>
- Chick, R. C. 2010a. Stock enhancement of local populations of blacklip abalone (*Haliotis rubra* Leach) in new South Wales, Australia. *PhD thesis, James Cook University*:1–260.
- Chick, R. C. 2010b. Batch-tagging blacklip abalone (*Haliotis rubra*) for identification of hatchery- reared individuals on natural coastal reefs in New South Wales, Australia. *Journal of Shellfish Research* 29:209–215.
- Chick, R. C., Worthington, D. G. & Kingsford, M. J. 2013. Restocking depleted wild stocks- long-term survival and impact of released blacklip abalone (*Haliotis rubra*) on depleted wild populations in New South Wales, Australia. *Reviews in Fisheries Science* 21:321–340. <https://doi.org/10.1080/10641262.2013.837278>
- Cloete, A. 2009. Profitability analysis of abalone farming in Port Nolloth, in the Northern Cape Province, South Africa. *The united nations University*:1–35.

- Collin, R. & Voltzow, J. 1998. Initiation, calcification, and form of larval 'Archaeogastropod' shells. *Journal of Morphology* 235:77–89.
[https://doi.org/10.1002/\(SICI\)1097-4687\(199801\)235:1<77::AID-JMOR6>3.0.CO;2-L](https://doi.org/10.1002/(SICI)1097-4687(199801)235:1<77::AID-JMOR6>3.0.CO;2-L)
- Cook, P. A. 2014. The Worldwide Abalone Industry. *Modern Economy* 05:1181–1186.
<https://doi.org/10.4236/me.2014.513110>
- Cook, P. A. 2016. Recent trends in worldwide abalone production. *Journal of Shellfish Research* 35:581–583. <https://doi.org/10.2983/035.035.0302>
- Cook, P. A. 2023. Worldwide abalone production: an update. *New Zealand Journal of Marine and Freshwater Research* 0:1–7.
<https://doi.org/10.1080/00288330.2023.2261869>
- De Viçose, G. C, Viera, M., Bilbao, A. & Izquierdo, M. 2010. Larval settlement of *Haliotis tuberculata* coccinea in response to different inductive cues and effect of larval density on settlement, early growth and survival. *Journal of Shellfish Research* 29:587–591.
- DAFF. 2017. Aquaculture Yearbook 2016, South Africa. Department of Agriculture, forestry and fisheries report, 1- 152. <https://doi.org/10.1007/BF03396777>
- Daume, S. 2006. The roles of bacteria and micro and macro algae in abalone aquaculture: A review. *Journal of Shellfish Research* 25:151–157. [https://doi.org/10.2983/0730-8000\(2006\)25](https://doi.org/10.2983/0730-8000(2006)25)
- Daume, S., Brand-Gardner, S. & Woelkerling, W. J. 1999a. Settlement of abalone larvae (*Haliotis laevis* Donovan) in response to non-geniculate coralline red algae (Corallinales, Rhodophyta). *Journal of Experimental Marine Biology and Ecology* 234:125–143. [https://doi.org/10.1016/S0022-0981\(98\)00143-9](https://doi.org/10.1016/S0022-0981(98)00143-9)
- Daume, S., Brand-Gardner, S. & Woelkerling, W. J. 1999b. Preferential settlement of abalone larvae: Diatom films vs. non-geniculate coralline red algae. *Aquaculture* 174:243–254. [https://doi.org/10.1016/S0044-8486\(99\)00003-4](https://doi.org/10.1016/S0044-8486(99)00003-4)
- Day, R., Williams, M. & Hawkes, G. 1995. A comparison of fluorochromes for marking abalone shells. *Marine and Freshwater Research* 46:599.
<https://doi.org/10.1071/mf9950599>

- De Waal, S. W. 2002. Factors influencing the ranching of the abalone species *Haliotis midae* along the Namaqualand coast of South Africa. *PhD thesis, University of Cape Town*:1–254.
- De Waal, S. W. & Cook, P. 2001. Quantifying the physical and biological attributes of successful ocean seeding sites for farm-reared juvenile abalone (*Haliotis midae*). *Journal of Shellfish Research* 20:857–861.
- DFFE. 2021. Aquaculture yearbook 2021, South Africa. Department of Forestry, Fisheries and the Environment. *Report*: 1-216.
- DFFE. 2023. Status of the South African Marine Fishery Resources. Department of Forestry, Fisheries and the Environment. *Report*: 1-84.
- Dixon, C. D., Day, R. W., Huchette, S. M. H. & Shepherd, S. A. 2006. Successful seeding of hatchery-produced juvenile greenlip abalone to restore wild stocks. *Fisheries Research* 78:179–185. <https://doi.org/10.1016/j.fishres.2005.11.023>
- Eads, C. & Layzer, J. B. 2002. How to pick your mussels out of a crowd: using fluorescence to mark juvenile freshwater mussels. *The University of Chicago press Journals* 21:476–486.
- Ersts, P.J. 2019. DotDotGoose (version 1.1.1). American museum of natural history, Centre for Biodiversity and Conservation.
https://biodiversityinformatics.amnh.org/open_source/dotdotgoose
- FAO. 2018. The State of World Fisheries and Aquaculture 2018 - Meeting the sustainable development goals. *Rome*: 1-227. Licence: CC BY-NC-SA 3.0 IGO.
- Fitzpatrick, M. P., Jeffs, A. G. & Dunphy, B. J. 2010. Identification of the optimal fluorochrome for marking larvae of the Pulmonate limpet *Siphonaria Australis*. *Journal of Shellfish Research* 29:941–944. <https://doi.org/10.2983/035.029.0427>
- Fitzpatrick, M. P., Jeffs, A. G. & Dunphy, B. J. 2013. Efficacy of calcein as a chemical marker of green-lipped mussel (*Perna canaliculus*) larvae and its potential use for tracking larval dispersal. *Aquaculture Research* 44:345–353.
<https://doi.org/10.1111/j.1365-2109.2011.03034.x>

- Fox, J. & Weisberg, S. 2019. An R companion to applied regression, third edition. *Thousand Oaks CA: Sage*.
- Freeman, K. A. 2001a Aquaculture and related biological attributes of abalone species in Australia – a review. *Fisheries Research Report* 128:1–40.
- Gallardo, W. G. & Buen, S. M. A. 2003. Evaluation of mucus, *Navicula*, and mixed diatoms as larval settlement inducers for the tropical abalone *Haliotis asinina*. *Aquaculture* 221:357–364. [https://doi.org/10.1016/S0044-8486\(03\)00121-2](https://doi.org/10.1016/S0044-8486(03)00121-2)
- Gapasin, R. S. J. & Polohan, B. B. 2004. Induction of larval settlement and metamorphosis in the donkey-ear abalone, *Haliotis asinina* Linnaeus, by chemical cues. *Hydrobiologia* 519:9–17.
- Genade, A. B., Hirst, A. L. & Smit, C. J. 1988. Observations on the spawning, development and rearing of the South African abalone *Haliotis midae* Linn. *South African Journal of Marine Science* 6:3–12. <https://doi.org/10.2989/025776188784480465>
- Gerrity, S. & Schiel, D. R. 2024. Assessing methods of enhancement for New Zealand blackfoot abalone (*Haliotis iris*) populations affected by mass mortality. *New Zealand Journal of Marine and Freshwater Research*:1–29.
- Glazer, R. 2005. A model for decoupling emigration from mortality in recapture surveys of slow-moving benthic marine gastropods. *Journal of Shellfish Research* 24 (2): 387–392. [http://dx.doi.org/10.2983/0730-8000\(2005\)24\[387:AMFDEF\]2.0.CO;2](http://dx.doi.org/10.2983/0730-8000(2005)24[387:AMFDEF]2.0.CO;2)
- Godfrey, B. P. 2003. The potential of abalone stock enhancement in the Eastern Cape province of South Africa. *MSc Thesis, Rhodes University*:1–170. <http://core.ac.uk>
- Goodsell, P. J., Underwood, A. J., Chapman, M. G. & Heasman, M. P. 2006. Seeding small numbers of cultured black-lip abalone (*Haliotis rubra* Leach) to match natural densities of wild populations. *Marine and Freshwater Research* 57:747–756. <https://doi.org/10.1071/MF06039>
- Gordon, N., Shpigel, M., Harpaz, S., Lee, J. J. & Neori, A. 2004. The settlement of abalone (*Haliotis discus hannai* Ino) larvae on culture layers of different diatoms. *Journal of Shellfish Research* 23:1–36.

- Goschen, W. S., Schumann, E. H., Bernard, K. S., Bailey, S. E. & Deyzel, S. H. P. 2012. Upwelling and ocean structures off Algoa Bay and the south-east coast of South Africa. *African Journal of Marine Science* 34:525–536.
<https://doi.org/10.2989/1814232X.2012.749810>
- Grubert, M. A. & Ritar, A. J. 2005. The effect of temperature and conditioning interval on the spawning success of wild-caught blacklip (*Haliotis rubra*, Leach 1814) and greenlip (*H. laevigata*, Donovan 1808) abalone. *Aquaculture Research* 36:654–665.
<https://doi.org/10.1111/j.1365-2109.2005.01269.x>
- Hadfield, M. & Paul, V. 2001. Natural chemical cues for settlement and metamorphosis of marine-invertebrate larvae. McClintock, K.B., Baker, B.J (eds) in Marine chemical ecology. *CRC Press LLC, New York*, 431–461.
<https://doi.org/10.1201/9781420036602.ch13>
- Hamasaki, K. & Kitada, S. 2008. The enhancement of abalone stocks: Lessons from Japanese case studies. *Fish and Fisheries* 9:243–260. <https://doi.org/10.1111/j.1467-2979.2008.00280.x>
- Hansen, S. C. 2006. Restoring endangered northern abalone (*Haliotis kamtschatkana*) population in British Colombia, Canada, using hatchery-raised individuals. *MSc Thesis, Thompson Rivers University*:1–179.
- Hansen, S. C. & Gosselin, L. A. 2016. Are hatchery-reared abalone naïve of predators? Comparing the behaviours of wild and hatchery-reared northern abalone, *Haliotis kamtschatkana* (Jonas, 1845). *Aquaculture Research* 47:1727–1736.
<https://doi.org/10.1111/are.12627>
- Hart, A. M. & Gorfine, H. K. 1997. Abundance estimation of blacklip abalone (*Haliotis rubra*) II. A comparative evaluation of catch-effort, change-in-ratio, mark-recapture and diver-survey methods. *Fisheries Research* 29:171–183.
- Hauck, M. & Sweijd, N. A. 2017. A case study of abalone poaching in South Africa and its impact on fisheries management. *Green Criminology*:223–231.
<https://doi.org/10.4324/9781315093390-11>

- Heasman, M., Chick, R., Savva, N., Worthington, D., Brand, C., Gibson, P. & Diemar, J. 2004. Enhancement of populations of abalone in NSW using hatchery-produced seed. *NSW Fisheries Final Report Series* 62:1–293.
- Heasman, M. & Savva, N. 2007. Manual for intensive hatchery production of abalone. *NSW Department of Primary Industries*:1–27.
- Henry, N. R. 1995. Studies related to the artificial spawning and culture of the abalone, *Haliotis midae* Linne, 1785. *MSc Thesis, University of Cape Town*:1–141.
- Hindrum, S. M., Burke, C. & Edwards, S. 2001. Growth reduction in greenlip (*Haliotis laevis*) and blacklip (*H. rubra*) abalone resulting from chronic exposure to sublethal combination of elevated ammonia and low dissolved oxygen levels. *Environmental requirements of abalone*:48–57.
- Hothorn, T., Bretz, F. & Westfall, P. 2008. Simultaneous inference in general parametric models. *Biometrical Journal* 50:346–363. <https://doi.org/10.1002/bimj.200810425>
- Huggett, M. J., Nys, R. De, Williamson, J. E., Heasman, M. & Steinberg, P. D. 2005. Settlement of larval blacklip abalone, *Haliotis rubra*, in response to green and red macroalgae. *Marine Biology* 147:1155–1163. <https://doi.org/10.1007/s00227-005-0005-6>
- Kaehler, S. & McQuaid, C. D. 1999. Use of the fluorochrome calcein as an in-situ growth marker in the brown mussel *Perna perna*. *Marine Biology* 133:455–460. <https://doi.org/10.1007/s002270050485>
- Kang, K. H., Kim, B. H. & Kim, J. M. 2004. Induction of larval settlement and metamorphosis of the abalone, *Haliotis discus hannai* larvae using bromomethane and potassium chloride. *Aquaculture* 230:249–259. Elsevier. [https://doi.org/10.1016/S0044-8486\(03\)00440-X](https://doi.org/10.1016/S0044-8486(03)00440-X)
- Kheder, B., Moal, J. & Robert, R. 2010. Impact of temperature on larval development and evolution of physiological indices in *Crassostrea gigas*. *Aquaculture* 309:286–289.
- Leighton, D. 1972. Laboratory observations on the early growth of the abalone, *Haliotis sorenseni*, and the effect of temperature on larval development and settling success. *Fishery Bulletin* 70:373–381.

- Leighton, D. 1974. The influence of temperature on larval and juvenile growth in three species of south California abalones. *Fishery bulletin* 72: 1137-1145
- Leighton, P. 2008. Abalone Hatchery Manual. *Irish Sea Fisheries Board*:1–89.
- Li, H., Wei, L., Zhang, G., Cai, Z., Cai, G., Chang, Y. & Xing, K. 2006. Enhancement of larval settlement and metamorphosis through biological and chemical cues in the abalone *Haliotis diversicolor supertexta*. *Aquaculture* 258:416–423.
<https://doi.org/10.1016/j.aquaculture.2006.04.013>
- Liu, B., Dong, B., Tang, B., Zhang, T. & Xiang, J. 2006. Effect of stocking density on growth, settlement and survival of clam larvae, *Meretrix meretrix*. *Aquaculture* 258:344–349. <https://doi.org/10.1016/j.aquaculture.2006.03.047>
- Lopez, L. M., Tyler, P. A. & Viana, M. T. 1998. The effect of temperature and artificial diets on growth rates of juvenile *Haliotis tuberculata* (Linnaeus, 1758). *Journal of Shellfish Research* 17:657–662.
- Lucas, T., Palmer, P. J., Wang, S., Scoones, R. & O'Brien, E. 2008. Marking the shell of the saucer scallop *Amusium balloti* for sea ranching using oxytetracycline, calcein and alizarin red S. *Journal of Shellfish Research* 27:1183–1188.
- Madikiza, L. 2012. Review and analysis of the status of abalone (*Haliotis midae*) fishery in South Africa. *United Nations University Fisheries Training Programme*:1-44.
<http://www.unuftp.is/static/fellows/document/liwalam12prf.pdf>
- Malzahn, A. M., Clemmesen, C. & Rosenthal, H. 2003. Temperature effects on growth and nucleic acids in laboratory-reared larval coregonid fish. *Marine Ecology Progress Series* 259:285–293. <https://doi.org/10.3354/meps259285>
- Maxwell, P. D. I. 2014. Scanning electron microscopy of the early life stages of the New Zealand yellowfoot paua, *Haliotis australis* and factors affecting settlement. *MSc Thesis, University of Otago*:1–128.
- Mayfield, S., Mcgarvey, R., Gorfine, H. K., Peeters, H., Burch, P. & SHARMA, S. 2011. Survey estimates of fishable biomass following a mass mortality in an Australian molluscan fishery. *Journal of Fish Diseases* 34:287–302. <https://doi.org/10.1111/j.1365-2761.2011.01241.x>

- McCarroll, R. J., Scott, T., King, E. V., Masselink, G., Ciotti, B. J., Cloete, A., Steyn, P. P., Britz, P., Vine, N. G., Deyzel, S. H. P., Witte, A. & Clark, R. 2021. Modelling regional and local-scale larval seeding strategies for abalone (*H. midae*) ranching in South Africa. *Aquaculture* 540:1–37. <https://doi.org/10.1016/j.aquaculture.2021.736668>
- McCormick, T. B., Herbinson, K., Mill, T. S. & Altick, J. 1994. A review of abalone seeding possible significance and a new seeding device. *Bulletin of Entomological Research* 55:680–693.
- McCormick, T. B., Navas, G., Buckley, L. M. & Biggs, C. 2016. Effect of temperature, diet, light, and cultivation density on growth and survival of larval and juvenile white abalone *Haliotis sorenseni* (Bartsch, 1940). *Journal of Shellfish Research* 35:981–992. <https://doi.org/10.2983/035.035.0421>
- McShane, P.E. 1992. Early life history of abalone: a review. Shepherd, S.A., Tegner, M. J., Guzman del Proo, S.A. (eds), *Abalone of the world: biology, fisheries and culture*. Oxford, UK: Blackwell: 120–138.
- Mills-Orcutt, K. A., Bouma, J. V & Donovan, D. A. 2020. Outplanting larval pinto abalone *Haliotis kamtschatkana kamtschatkana* (Jonas) as a recovery tool in the Salish sea. *Journal of Shellfish Research* 39:381–388. <https://doi.org/10.2983/035.039.0220>
- Miyake, Y., Kimura, S., Horii, T. & Kawamura, T. 2017. Larval dispersal of abalone and its three modes: A review. *Journal of Shellfish Research* 36:157–167. <https://doi.org/10.2983/035.036.0116>
- Moran, A. L. 2000. Calcein as a marker in experimental studies newly-hatched gastropods. *Marine Biology* 137:893–898. <https://doi.org/10.1007/s002270000390>
- Moran, A. L. & Marko, P. B. 2005. A simple technique for physical marking of larvae of marine bivalves. *Journal of Shellfish Research* 24:567–571. [https://doi.org/10.2983/0730-8000\(2005\)24\[567:ASTFPM\]2.0.CO;2](https://doi.org/10.2983/0730-8000(2005)24[567:ASTFPM]2.0.CO;2)
- Morse, A. N. C. 1991. How do planktonic larvae know where to settle? *American Scientist* 79:154–167.

- Moss, G. A. 1999. Factors affecting settlement and early post - settlement survival of the New Zealand abalone *Haliotis australis*. *New Zealand Journal of Marine and Freshwater Research* 33:271–278. <https://doi.org/10.1080/00288330.1999.9516876>
- Moss, G. A. & Tong, L. J. 1992a. Techniques for enhancing larval settlement of the abalone, *Haliotis iris*, on artificial surfaces on artificial surfaces. *New Zealand Journal of Marine and Freshwater Research* 26:75–79. <https://doi.org/10.1080/00288330.1992.9516502>
- Moss, G. A. & Tong, L. J. 1992b. Effect of stage of larval development on the settlement of the abalone, *Haliotis iris*. *New Zealand Journal of Marine and Freshwater Research* 26:69–73. <https://doi.org/10.1080/00288330.1992.9516501>
- Moriarty, L. A. 2019. Inshore benthic communities of the Port Elizabeth abalone ranching concession area. MSc Thesis, Nelson Mandela University: 1- 86.
<http://hdl.handle.net/10948/41975>
- Muller, L. 2003. Effect of illumination, shading and temperature on the settlement, survival and growth of juvenile abalone (*Haliotis midae*). MSc Thesis, University of Cape Town:1–100.
- Mzozo, Z. B., Hugo, S. & Vine, N. G. 2021. Effect of temperature on the development and settlement of the abalone larvae *Haliotis midae*: consideration for larvae hatchery management and stock enhancement. *Journal of Shellfish Research* 40:119–125.
<https://doi.org/10.2983/035.040.0111>
- Mzozo, Z. B., Hugo, S. & Vine, N. G. 2023. The use of chemical and biological settlement cues in enhancing the larval settlement of abalone (*Haliotis midae*): Implications for hatcheries and ocean ranching. *Journal of the World Aquaculture Society*:1–16.
<https://doi.org/10.1111/jwas.13001>
- Nosrolahi, A. 2012. Stress ecology: interactive effect of temperature and salinity on early life stages of barnacle, *Amphibalanus improvisus* -. PhD thesis, Christian-Albrechts University:1–153.
- Olla, B. L., Davis, M. W., Ryer, C. H. 1998. Understanding how the hatchery environment represses or promotes the development of behavioral survival skills. *Bulletin of Marine Science* 62 (2): 531-550.

- Onitsuka, T., Kawamura, T. & Horii, T. 2010. Reproduction and early life ecology of abalone *Haliotis diversicolor* in Sagami Bay, Japan. *Japan Agricultural Research Quarterly* 44:375–382. <https://doi.org/10.6090/jarq.44.375>
- Pang, S. J., Zhang, Z. H., Bao, Y. & Gao, S. Q. 2006. Settling abalone veliger larvae in a free-swimming microalgal culture. *Aquaculture* 258:327–336. <https://doi.org/10.1016/j.aquaculture.2006.03.028>
- Pedroso, F. L. 2017. Effects of elevated temperature on the different life stages of tropical mollusk, donkey's ear abalone (*Haliotis asinina*). *AACL Bioflux* 10:1421–1427.
- Pirker, J. G. & Schiel, D. R. 1993. Tetracycline as a fluorescent shell-marker in the abalone *Haliotis iris*. *Marine Biology: International Journal on Life in Oceans and Coastal Waters* 116:81–86. <https://doi.org/10.1007/BF00350734>
- Preece, P. A., Shepherd, S. A., Clarke, S. M. & Keesing, J. K. 1997. Abalone stock enhancement by larval seeding: Effect of larval density on settlement and survival. *Molluscan Research* 18:265–273. <https://doi.org/10.1080/13235818.1997.10673700>
- Preece, P. A., Shepherd, S. A., Clarke, S. M. & Keesing, J. K. 2000. Feasibility of enhancing abalone stocks by larval seeding. *South Australian research and development institute*:1–49.
- Prince, J. D., Sellers, T. L., Ford, W. B. & Talbot, S. R. 1987. Experimental evidence for limited dispersal of haliotid larvae (genus *Haliotis*; Mollusca: Gastropoda). *Journal of Experimental Marine Biology and Ecology* 106:243–263. [https://doi.org/10.1016/0022-0981\(87\)90096-7](https://doi.org/10.1016/0022-0981(87)90096-7)
- Purcell, S. W. & Blockmans, B. F. 2009. Effective fluorochrome marking of juvenile sea cucumbers for sea ranching and restocking. *Aquaculture* 296:263–270. <https://doi.org/10.1016/j.aquaculture.2009.08.027>
- R Core Team. 2021. A language and environment for statistical computing. in. *R Foundation for Statistical Computing, Vienna, Austria*.
- Raemaekers, S., Hauck, M., Bürgener, M., Mackenzie, A., Maharaj, G., Plagányi, É. E. & Britz, P. J. 2011. Review of the causes of the rise of the illegal South African abalone

- fishery and consequent closure of the rights-based fishery. *Ocean and Coastal Management* 54:433–445. <https://doi.org/10.1016/j.ocecoaman.2011.02.001>
- Raemaekers, S. J. P. N. & Britz, P. J. 2009. Profile of the illegal abalone fishery (*Haliotis midae*) in the Eastern Cape Province, South Africa: Organised pillage and management failure. *Fisheries Research* 97:183–195. <https://doi.org/10.1016/j.fishres.2009.02.003>
- Rahn, B. A. & Perren, S. M. 1971. Xylenol orange, a fluorochrome useful in polychrome sequential labeling of calfiying tissue. *Stain Technology* 4:125–129.
- Robert, R. 2001. A review of settlement cues for larval abalone (*Haliotis* spp.). *Journal of Shellfish Research* 20:571–586. [https://doi.org/10.2983/0730-8000\(2007\)26\[675:P\]2.0.CO;2](https://doi.org/10.2983/0730-8000(2007)26[675:P]2.0.CO;2)
- Roberts, R. D., Keys, E. F., Prendeville, G. & Pilditch, C. A. 2007. Viability of abalone (*Haliotis iris*) stock enhancement by release of hatchery-reared seed in Marlborough, New Zealand. *Journal of Shellfish Research* 26:697–703. [https://doi.org/10.2983/0730-8000\(2007\)26\[697:VOAHIS\]2.0.CO;2](https://doi.org/10.2983/0730-8000(2007)26[697:VOAHIS]2.0.CO;2)
- Roberts, R. D. & Lapworth, C. 2001. Effect of delayed metamorphosis on larval competence and post larval survival and growth, in the abalone *Haliotis iris* Gmelin. *Journal of Experimental Marine Biology and Ecology* 258:1–13.
- Rogers-Bennett, L., Dondanville, R. F., Catton, C. A., Juhasz, C. I., Horii, T. & Hamaguchi, M. 2016. Tracking Larval, Newly Settled, and Juvenile Red Abalone (*Haliotis rufescens*) Recruitment in Northern California. *Journal of Shellfish Research* 35:601–609.
- Sales, J. & Britz, P. J. 2001. Research on abalone (*Haliotis midae* L.) cultivation in South Africa. *Aquaculture Research* 32:863–874. <https://doi.org/10.1046/j.1365-2109.2001.00629.x>
- Samsing, F., Oppedal, F., Dalvin, S., Johnsen, I., Vågseth, T. & Dempster, T. 2016. Salmon lice (*Lepeophtheirus salmonis*) development times, body size, and reproductive outputs follow universal models of temperature dependence. *Canadian Journal of Fisheries and Aquatic Sciences* 73:1841–1851.

- Scharf, I., Braf, H., Ifrach, N., Rosenstein, S. & Subach, A. 2015. The effects of temperature and diet during development, adulthood and mating on reproduction in the red flour beetle. *Plos One*:1–15.
- Searle, T., Roberts, R. D. & Lokman, P. M. 2006. Effects of temperature on growth of juvenile blackfoot abalone, *Haliotis iris* Gmelin. *Aquaculture Research* 37:1441–1449. <https://doi.org/10.1111/j.1365-2109.2006.01580.x>
- Shepherd, S. A., Lowe, D. & Partington, D. 1992. Studies on southern Australian abalone (genus *Haliotis*) XIII: larval dispersal and recruitment. *Journal of Experimental Marine Biology and Ecology* 164:247–260. [https://doi.org/10.1016/0022-0981\(92\)90178-D](https://doi.org/10.1016/0022-0981(92)90178-D)
- Slattery, M. 1987. Settlement and Metamorphosis of red abalone (*Haliotis rufescences*) larvae: A critical examination of mucus, diatoms and Y_aminobutyric acid (GABA) as inductive sunstrates. *MSc Thesis, San Jose State University*:1–39.
- Slattery, M. 1992. Larval settlement and juvenile survival in the red abalone (*Haliotis rufescens*), an examination of inductive cues and substrate selection. *Aquaculture* 102:143–153. [https://doi.org/10.1016/0044-8486\(92\)90296-W](https://doi.org/10.1016/0044-8486(92)90296-W)
- Smit, A. J., Roberts, M., Anderson, R. J., Dufois, F., Dudley, S. F. J., Bornman, T. G., Olbers, J. & Bolton, J. J. 2013. A coastal seawater temperature dataset for biogeographical studies: large biases between in situ and remotely-sensed data sets around the coast of South Africa. *Plos One* 8:1–13. <https://doi.org/10.1371/journal.pone.0081944>
- South African Government Gazette. 2010. Department of agriculture, forestry and fisheries marine living resources act, 1998 (act no. 18 of 1998). *Report*:8–44.
- Stephens, S. A., Broekhuizen, N., Macdiarmid, A. B., Lundquist, C. J., Mcleod, L. & Haskew, R. 2006. Modelling transport of larval New Zealand abalone (*Haliotis iris*) along an open coast. *Marine and Freshwater Research* 57:519–532. <https://doi.org/10.1071/MF06020>
- Stewart, P., Soonklang, N., Stewart, M. J., Wanichanon, C., Hanna, P. J., Poomtong, T. & Sobhon, P. 2008. Larval settlement of the tropical abalone, *Haliotis asinina* Linnaeus, using natural and artificial chemical inducers. *Aquaculture Research*:1181–1189. <https://doi.org/10.1111/j.1365-2109.2008.01982.x>

- Stickney, R. 2005. Aquaculture: An introductory text. *CABI Publishing*. 1–83 pp.
- Stuart, A. A. J. & Smith, D. A. 1992. Use of the fluorochromes xylenol orange, calcein green, and tetracycline to document bone deposition and remodeling in healing fractures in chickens. *American Association of Avian Pathologists* 36:447–449.
- Sweijd, N., Snethlage, Q., Harvey, D., Cook, P. 1998. Fisheries-Experimental abalone (*Haliotis midae*) seeding in South Africa. *Journal of Shellfish Research* 17(3): 897-904.
- Takami, H., Kawamura, T., Yamashita, Y. 1997. Survival and growth rates of post-larval abalone *Haliotis discus hannai* fed conspecific trail mucus and/or benthic diatom *Cocconeis scutellum var. parva*. *Aquaculture* 152: 129–138.
- Tebben, J., Motti, C. A., Siboni, N., Tapiolas, D. M., Negri, A. P., Schupp, P. J., Kitamura, M., Hatta, M., Steinberg, P. D. & Harder, T. 2015. Chemical mediation of coral larval settlement by crustose coralline algae. *Scientific Reports* 5:1–11.
<https://doi.org/10.1038/srep10803>
- Tegner, M. J. 1992. Brood-stock transplants as an approach to abalone stock enhancement. Shepherd, S.A., Tegner, M.J., Guzman del Proo S.A. (eds), Abalone of the world: biology, fisheries and culture. Oxford, UK: Blackwell, 461–473.
- Todd, C. D., Bentley, M. G. & Jonathan, N. H. 1991. Larval metamorphosis of the opisthobranch mollusc *Adalaria proxima* (Gastropoda: Nudibranchia): The effects of choline and elevated potassium ion concentration. *Journal of the Marine Biological Association of the United Kingdom* 71:53–72.
<https://doi.org/10.1017/S0025315400037395>
- Tong, L. J., Moss, G. A. & Illingworth, J. 1987. Enhancement of a natural population of the abalone, *Haliotis iris*, using cultured larvae. *Aquaculture* 62:67–72.
[https://doi.org/10.1016/0044-8486\(87\)90185-2](https://doi.org/10.1016/0044-8486(87)90185-2)
- Troell, M. D., Robertson-Andersson, R. J., Anderson, R. J., Bolton, J. J., Maneveldt, G., Halling, C. & Probyn, T. 2006. Abalone farming in South Africa: an overview with perspectives on kelp resources, abalone feed, potential for on-farm seaweed production and socio-economic importance. *Aquaculture* 257:266–281.
<https://doi.org/10.1016/j.aquaculture.2006.02.066>

- Uchino, K., Simizu, T., Tanaka, T. & Shibata, T. 2004. Abalone resource decline and recovery attempt in Chiba prefecture, Japan. *Journal of Shellfish Research* 23:1219–1222.
- Uggeri, J., Gatti, R., Belletti, S., Scandroglio, R., Corradini, R., Rotoli, B. M. & Orlandini, G. 2004. Calcein-AM is a detector of intracellular oxidative activity. *Histochemistry and Cell Biology* 122:499–505. <https://doi.org/10.1007/s00418-004-0712-y>
- van der Geest, M., Van Gils, J. A., Van Deer Meer, J., Olff, H. & Piersma, T. 2011a. Suitability of calcein as an in situ growth marker in burrowing bivalves. *Journal of Experimental Marine Biology and Ecology* 399:1–7. <https://doi.org/10.1016/j.jembe.2011.01.003>
- van der Merwe, E. 2009. Toward best management practices for the growth of the abalone *Haliotis midae* Linnaeus on a commercial South African abalone farm. *MSc Thesis, University of Western Cape*:1–122.
- Visser-Roux, A. 2011. Reproduction of the South African abalone *Haliotis midae*. *PhD Thesis, University of Stellenbosch*:1–102.
- White, H. I., Hecht, T. & Potgieter, B. 1996. The effect of four anaesthetics on *Haliotis midae* and their suitability for application in commercial abalone culture. *Aquaculture* 140:145–151. [https://doi.org/10.1016/0044-8486\(95\)01185-4](https://doi.org/10.1016/0044-8486(95)01185-4)
- Witte, W. 2017. Abalone stock enhancement at Cape Recife, Eastern Cape province of South Africa. *MSc Thesis, Rhodes University*:1–168.
- Witte, A. 2018. Recruitment and population dynamics of seeded and wild abalone. Rhodes University.
- Wood, A. 1993. Aspects of the biology and ecology of the South African abalone *Haliotis midae* Linnaeus, 1758 (Mollusca: Gastropoda) along the Eastern Cape and Ciskei coast. *MSc Thesis, Rhodes University* : 1- 168
- Yool, A. J., Grau, S. M., Hadfield, M. G., Jensen, R. A., Markell, D. A. & Morse, D. E. 1986. Excess potassium induces larval metamorphosis in four marine invertebrate species. *The Biological Bulletin* 170:255–266. <https://doi.org/10.2307/1541807>

Yu, X., Yan, Y. & Li, H. 2010. The effect of chemical cues on larval settlement of the abalone, *Haliotis diversicolor* supertexta. *Journal of the World Aquaculture Society* 41:626–632. <https://doi.org/10.1111/j.1749-7345.2010.00403.x>



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Appendix 1 Ethical clearance certificate



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ETHICAL CLEARANCE CERTIFICATE AREC-150311-008

Certificate Reference Number: VIN051SMZO01

Project title: **Factors influencing larval settlement of abalone (*Haliotis midae*): towards the ranching of abalone larvae**

Nature of Project PhD in Zoology

Principal Researcher: Ziyanda Brightness Mzozo

Supervisor: Prof. N. Vine

On behalf of the University of Fort Hare's Animal Research Ethics Committee (AREC) I hereby give ethical approval in respect of the undertakings contained in the above-mentioned project and research instrument(s). Should any other instruments be used, these require separate authorization. The Researcher may therefore commence with the research as from the date of this certificate, using the reference number indicated above.

Special conditions: Please submit a copy of the permit required to release farmed abalone into the ocean for the University of Fort Hare Animal Research Ethics Committee's records.

Please note that the AREC must be informed immediately of

- Any material change in the conditions or undertakings mentioned in the document;
- Any material breaches of ethical undertakings or events that impact upon the ethical conduct of the research.

The Principal Researcher must report to the AREC in the prescribed format, where applicable, annually, and at the end of the project, in respect of ethical compliance. The AREC retains the right to

- Withdraw or amend this Ethical Clearance Certificate if
 - Any unethical principal or practices are revealed or suspected;
 - Relevant information has been withheld or misrepresented;
 - Regulatory changes of whatsoever nature so require;
 - The conditions contained in the Certificate have not been adhered to.
- Request access to any information or data at any time during the course or after completion of the project.
- In addition to the need to comply with the highest level of ethical conduct principle investigators must report back annually as an evaluation and monitoring mechanism on the progress being made by the research. Such a report must be sent to the Dean of Research's office.

The Animal Research Ethics Committee wishes you well in your research.

Yours sincerely



Dr. Craig Tambling
AREC Chairperson

29 November 2018

Appendix 2 Published research article

Journal of Shellfish Research, Vol. 40, No. 1, 119–125, 2021.

EFFECT OF TEMPERATURE ON THE DEVELOPMENT AND SETTLEMENT OF THE ABALONE LARVAE *HALIOTIS MIDAE*: CONSIDERATIONS FOR ABALONE HATCHERY MANAGEMENT AND STOCK ENHANCEMENT

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ABSTRACT Abalones are poikilotherms, and their rate of development is intrinsically linked with temperature. Understanding the effect of temperature on development is key for the successful production planning in abalone hatcheries and the reseeded of cultured abalone larvae for stock enhancement. This study investigates the effect of temperature on the development of the abalone larvae *Haliotis midae*. Newly hatched trochophore larvae were raised at temperatures of 14°C, 16°C, 18°C, 20°C, and 22°C until settlement. The temperature had a significant effect on the larval development—most rapid at 22°C [3 days and 65 degree-days (DD), °C day] and slowest at 14°C (6.7 days and 95 DD). Larval settlement was highest at 22°C (43.44% ± 3.73%, larvae). Larvae should preferably be seeded at temperatures between 20°C and 22°C as this will potentially increase settlement, thereby reducing larval dispersion outside of the seeding area.

KEY WORDS: abalone larvae, *Haliotis midae*, cumulative degree-days, aquaculture

INTRODUCTION

Abalones are poikilotherms and have a limited thermal range with their growth and other biological processes predominately determined by the temperature of their surrounding environment (Lopez et al. 1998, Searle et al. 2006, Kheder et al. 2010, Pedroso 2017). Within the limits of a lower development threshold and an upper lethal temperature, an increase in temperature results in higher enzymatic reaction rates which support a faster growth rate (Muller 2003, Pedroso 2017). Temperature, therefore, influences abalones biological rates such as spawning, developmental rates, and life span (Kheder et al. 2010). The exposure to temperatures beyond physiological tolerance limits can be stressful, fatal, or slow down the growth (Muller 2003, Pedroso 2017). The larval period of *Haliotis midae* typically takes between 4 and 10 days, depending on the water temperature (Visser-Roux 2011).

In South Africa, the large-scale illegal harvesting of the wild abalones *Haliotis midae* has caused a rapid decline of wild abalones stock (De Waal 2002), with stocks considered to be heavily depleted (DAFF Report 2016). To rebuild the abalones stock fisheries, stock enhancement has been recommended as a fishery management tool to rehabilitate wild stocks using cultured juveniles (Godfrey 2003). Information on stock enhancement using hatchery-raised *H. midae* juvenile abalones has been reported in several articles (De Waal & Cook 2001, Godfrey 2003) but has not been investigated using *H. midae* larvae.

Although temperature can be easily manipulated in the hatchery when rearing abalone larvae, the temperature of the natural environment may vary daily and seasonally (Muller 2003). Abalones *Haliotis midae* occurs from St. Helena Bay on the West Coast of South Africa to the north of Port St. Johns on the East Coast (Visser-Roux 2011), where the coastline temperatures range from an average of 13°C–25°C (Smit et al. 2013). Therefore, understanding the effect of temperature on the development and settlement of larvae is a critical first step in

developing a restoration effort to seed farm-spawned larvae into the ocean.

This study was designed to test the effect of five different temperatures (14°C–22°C) on the development and settlement of abalone larvae (*Haliotis midae*) that were reared at the hatchery. The temperature treatments that were used in this study are within the natural range for *H. midae*. To be able to compare the temperature effect on development, the data were analyzed in relation to degree-days (DD). The information can be used to model larval development, thereby estimating larval competence to settle across a range of temperatures. The findings can also be used for effective planning of production in *H. midae* hatcheries.

MATERIALS AND METHODS

Five temperature treatments (14 ± 0.11°C, 16 ± 0.4°C, 18 ± 0.11°C, 20 ± 0.23°C, and 22 ± 0.02°C) with four replicates each were used to determine the effect of temperature on the developmental rate of abalone larvae until settlement. The experiment was conducted at Wild Coast Abalone (Pty) Ltd farm (32° 45' 05" S 28° 16' 27" E), South Africa. Twenty-five liter aquariums (filled to 23 L) were connected to a flow-through system, and temperature treatments were allocated randomly, with each aquarium receiving independent aeration and filtered (1 µm) UV-treated seawater at ±0.33 L/min. The photoperiod was set at 12L:12D. Ambient temperature was controlled with air conditioners and maintained at 16°C. The seawater from the sump was coming in at 16°C. Thermal glass heaters (Xilong Aquarium glass heater, AT-700–200 W) were used to increase the temperature in individual aquariums to 18°C, 20°C, and 22°C. A water chiller (Titan 2000, Aqua Medic) was used to reduce the temperature to 14°C. Throughout the experiment, the temperature in each aquarium was monitored daily (using a 0.1°C gradient thermometer) to verify that all temperatures were stable.

Fertilized abalone eggs (stage 1, see Table 1) were collected from the farm broodstock and hatched in 1-µm filtered seawater maintained at 17°C. Larvae were then transferred into the aquariums at approximately 50,000 (2.17 larvae/mL) newly

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TABLE 1.
Developmental stages of abalone larvae *Haliotis midae* (Genade et al. 1988).

No.	Life cycle stage	Developmental stage	Description
1	Egg	Fertilization	Fertilization, and discharge of the two polar bodies
2	Trochophore	Hatching	Initial larval shell secretion
3	Veliger	Pre-eye spot	The appearance of the pre-eye spot
4	Veliger	Retracted velum	Development of a short spine on the cephalic tentacle
5	Veliger	Crawling and settling	Development of the fourth tubule on the cephalic tentacle

hatched larvae (stage 2, see Table 1) per aquarium. For all treatments, the temperature was initially maintained at 16°C and then gradually increased or decreased over 8 h until the desired temperature in the aquarium was reached. This allowed the larvae to acclimate to each experimental temperature.

Larval development was determined by taking triplicate 10 mL water samples from each aquarium twice a day (approximately 8 AM and 3 PM) and identifying the larval developmental stage under a dissecting microscope (NSZ-606, Best Scope). Distinctive external and internal larval features were identified following descriptions by Genade et al. (1988) (Table 1). A developmental stage was considered to be attained when more than 50% of the larvae observed in a sample had the features associated with that stage; the time of this event from the start of the experiment was recorded.

Larvae were raised until settlement, which was defined as the attachment to a benthic substrate. Settlement was considered to be complete once there were no more free-swimming larvae detected in the aquarium water. Before the experiment, plastic sheets (15 × 15 cm = 450 cm² total surface area) were placed in outside tanks supplied with incoming seawater and allowed to develop a film of natural diatoms. On day four of the experiment, two sheets were suspended in each aquarium to help induce larval settlement. Once the larvae had settled, the sheet was briefly removed at around 8 AM daily and photographed (Canon camera EOS 2000D), and the number of settled larvae per side of the sheet was counted. In addition to settling on the plastic sheets, larvae also settled on the sides of the aquarium, but these were not counted. Instead, the total number was extrapolated based on the total surface area of the aquarium. The experiment was conducted for 10 days.

Temperature data loggers (UA-001-08, Onset HOBO data loggers) were placed into two aquariums of each treatment to record the temperature at 10-min intervals throughout the study period. The data collected from the loggers were used to calculate cumulative DD (°C day) as follows:

$$DD = \text{Time(days)} \times \text{Average temperature(°C)}.$$

Time was measured in 24 h days (n), whereas the average temperature was the average rearing temperature across n days.

STATISTICAL ANALYSIS

Observation of Developmental Stages

Although the experimental design had four replicates, these replicates are not relevant in this analysis because the stages were observed in all replicates at the same time (no variation

among replicates). To study the development rate of larvae in relation to temperature, a generalized linear model (R Core Team 2020) was used to examine how temperature determines the hour or DD at which each developmental stage is observed. With hour or DD as the response variable, separate slope coefficients (i.e., development rates) were obtained for each developmental stage by modeling the interaction effect of temperature with a factor variable representing the developmental stage. Although temperature treatment is essentially a factor with five levels in the experimental design, temperature was used as a continuous predictor variable in this model because there were too few data for a model with two interacting factor variables. Because both hours and DD are continuous variables that can only be positive, a Gamma distribution family was used. The overall significance of the predictors was expressed as an analysis of variance (ANOVA, R function “Anova” in the “car” package, Fox & Weisberg 2019).

Temporal Settlement of Larvae at Each Temperature

Exploratory analyses show that the number of larvae settling over time appears to be nonlinear (Fig. 1); however, preliminary analyses with nonlinear models (R function “nls,” R Core Team 2020) failed (e.g., the Lomolino function) or fitted the data very poorly (e.g., a logistic function), as the number of observations over time (maximum seven for 20°C and 22°C) was too few to reasonably fit a nonlinear function. Furthermore, the abundance of settled larvae observed at the conclusion of the experiment differs substantially among temperature treatments (Fig. 1).

Therefore, to study larval settlement over time at different temperatures, a linear random-effects model (R package “lme4,” Bates et al. 2015) with the Poisson distribution family was used to relate the number of larvae counted on the sheets to two random effects—hours (random slope) and temperature as a factor (random intercept). This model supplies a unique slope (rate of settlement) and intercept (abundance reached) for each temperature treatment. Even though this random-effects model is fairly complex, preliminary analyses showed that it fitted the response variable better (the lowest Akaike’s information criterion value) than other models where either or both hours and temperature were modeled as fixed effects. An analysis of deviance was used to obtain a Likelihood ratio chi-square value (hereafter χ^2) and approximate P value for hours and temperature, by comparing models with or without the relevant random effect.

RESULTS

The rate of larval development varied with temperature, suggesting that temperature plays a significant role in the

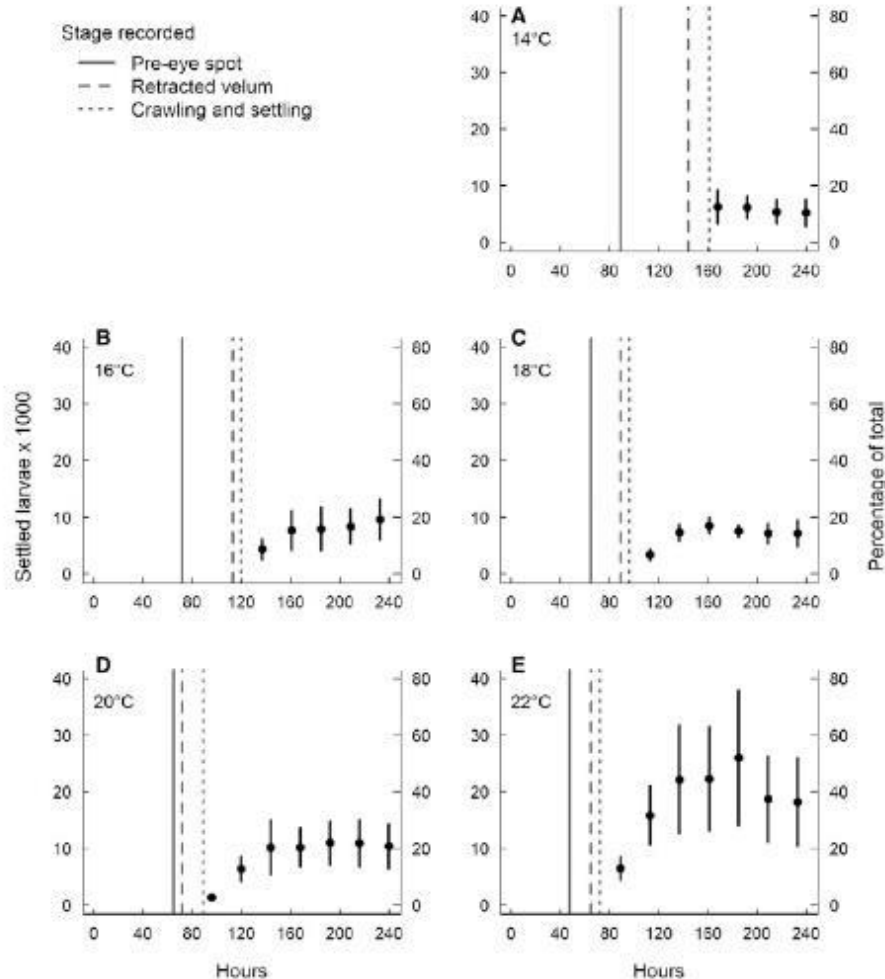


Figure 1. The estimated mean number ($\pm$ SD) of larvae settled. Equivalent percentage, out of ca 50,000 larvae initially added to each aquarium, is indicated on the right-hand y-axis. The vertical lines depict the observed hour at which greater than 50% of the larvae had reached each particular stage.

development of *Haliotis midae*. With each 2°C change in temperature, there was an increase in the rate of development. Temperature is a strong predictor of when a particular stage of development is reached, both when considering time (hours) until a stage is reached (ANOVA: $\chi^2 = 215.34$, $P < 0.0001$) and DD (ANOVA: $\chi^2 = 112.59$, $P < 0.0001$). Specifically, each developmental stage is observed significantly earlier, or at a lesser DD values, as temperature increases (Table 2, Fig. 2).

The results of developmental time showed that *Haliotis midae* larvae developed until they were competent to settle at a wide range of temperatures (14°C–22°C). The model of larval settlement at different temperatures is significantly influenced by both the random intercept term denoting overall settled larval abundance differences between temperatures ($\chi^2 = 33,788.4$, $P < 0.0001$) and the random slope term denoting different rates of settlement at different temperatures ($\chi^2 = 8,634.2$, $P < 0.0001$). The overall abundance of settled larvae differed substantially between treatments (Table 3, Fig. 1). For example, the maximum number of settled larvae observed at

22°C (on average about 52% of the total introduced at the beginning of the experiment) is more than twice the maximum number of settled larvae observed at 20°C (average 22% of the

TABLE 2.
Intercept with the slope coefficients* of the relationship between hours/DD and temperature at each developmental stage for *Haliotis midae* larvae.

	Hours	DD
Intercept	456.530 (342.916; 608.980)†	135.167 (101.932; 179.573)
Pre-eye spot	0.899 (0.885; 0.913)	0.946 (0.931; 0.961)
Retracted velum	0.915 (0.900; 0.930)	0.963 (0.948; 0.978)
Crawling and settling	0.924 (0.909; 0.939)	0.972 (0.956; 0.988)

DD, degree-day.

* Coefficients were back-transformed from the model's log scale.

† 95% confident interval in the brackets.

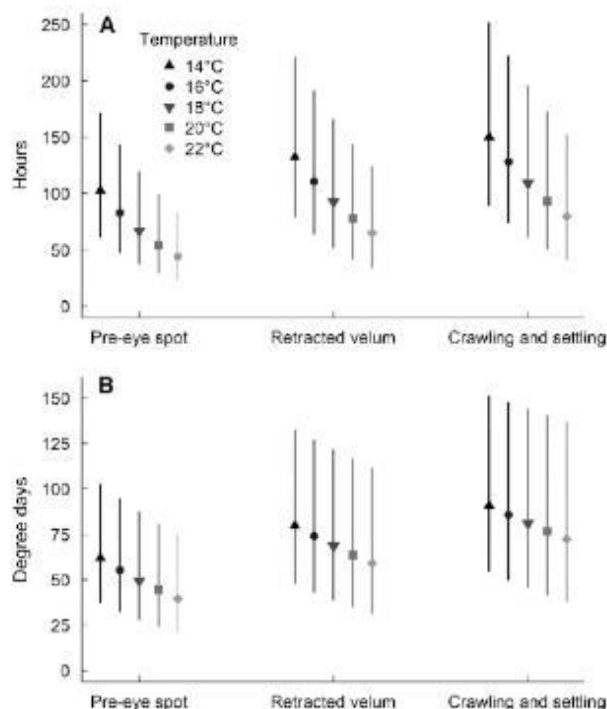


Figure 2. The predicted hour or degree-days at which each developmental stage of *Haliotis midae* larvae was observed at each temperature, based on generalized linear models. The error bars are 95% confidence intervals.

total). Larval settlement increased over time for all temperatures (back-transformed slope coefficient > 1), except at 14°C where it decreased over time (< 1) (Table 3, Fig. 1). Furthermore, for temperatures between 16°C and 22°C, the rate of the settlement was not consistently faster for warmer temperatures (the slope is not steeper with an increase in temperature, Table 3). It is likely, however, that these slope coefficients are not helpful to describe the effect of temperature because an increase in the number of settled larvae mainly happens at early stages before leveling out (Fig. 1), and the linear model cannot account for this nonlinear pattern. Therefore, the current model and data cannot be used to predict larval settlement in general. To create a predictive model, more observations over time are needed to allow a nonlinear model to be fitted.

TABLE 3.

Intercept* and slope coefficients* for the number of larvae (*Haliotis midae*) settled over time (hours) at each temperature.

Temperature (°C)	Intercept	Slope
14	1,107 (1,083, 1,131)†	0.881 (0.859; 0.904)
16	1,243 (1,226, 1,260)	1.318 (1.297; 1.339)
18	1,216 (1,202, 1,230)	1.148 (1.133; 1.162)
20	1,533 (1,518, 1,547)	1.337 (1.325; 1.350)
22	3,428 (3,406, 3,450)	1.175 (1.168; 1.182)

* Coefficients were back-transformed from the model's log scale.

† The lower and upper 95% confidence intervals in brackets.

By the end of the experiment, no larvae were swimming in the aquariums at temperatures 18°C, 20°C, and 22°C; however, at 14°C and 16°C, there were still 0.2 ± 0.06 and 0.15 ± 0.04 larvae/mL, respectively. Abalone larvae which had settled on the sheets in the 18°C, 20°C, and 22°C treatments had metamorphosed, whereas larvae in the 14°C and 16°C treatments had not.

DISCUSSION

When food is not a limiting factor, temperature has a significant impact on the rate of development of many organisms (Scharf et al. 2015). It controls the principal biochemical processes of metabolism and regulates developmental rates (Samsing et al. 2016). Because temperature controls abalones growth, it is considered to be an ecological resource and can be quantified using a DD approach (Chezik et al. 2014). The DD approach works best when describing the development in endogenous feeding larvae because the development is only controlled by age, temperature, and yolk quality (Malzahn et al. 2003). The DD approach assumes that there is a linear relationship between temperature and growth (Malzahn et al. 2003, Chezik et al. 2014). It allows for developmental comparison between temperatures by reducing the effect of age (days), meaning that larvae with the same age but not temperature history can be directly compared (Malzahn et al. 2003). In this study, the development of *Haliotis midae* larvae at different temperatures was compared using the DD approach. The data presented in this article show a significant influence of temperature on the development of *H. midae* larvae. Abalone larvae at the same age (days) had a faster developmental rate at a higher temperature (22°C) than at a lower temperature (14°C). Chezik et al. (2014) reported that the DD approach can be used to predict the growth of fish larvae as it outperforms calendar time—it directly measures the thermal opportunity and predicts growth at accuracy within 2 days. Therefore, the DD approach can be used for long-term planning in abalone farm hatcheries.

Temperature controls the principal biochemical processes of metabolism and regulates developmental rates (Samsing et al. 2016). The use of the DD approach to normalizing the data clearly showed the temperature dependence of the development of *Haliotis midae* larvae. In the present study, the developmental period for *H. midae* larvae to reach the various stages of development decreased with an increase in temperature across the range tested. The rate of larval development increased with temperature over the range of 14°C–22°C, with every 2°C change in the temperature significantly increasing the rate of development. Larvae cultured at 22°C completed development and were competent to settle in 3.7 days (80 DD), whereas those held at 14°C had just developed a pre-eye spot (Fig. 2). Similar results were observed by Muller (2003), Grubert and Ritar (2004), Onitsuka et al. (2010), Visser-Roux (2011), McCormick et al. (2016), and Pedrosa (2017), who reported that the developmental rate was most rapid at higher temperatures and slowest at lower temperatures. The results from this study showed that *H. midae* raised at 20°C completed development and were competent to settle in 4 days (77.88 DD). These findings contradict the results of Genade et al. (1988) who found that *H. midae* larvae raised at 20°C were competent to settle in 5 days. This could be attributed to possible genetic differences between the broodstock. The abalones broodstock used in this

study were F1 individuals from Wild Coast Abalone farm's hatchery, whereas Genade et al. (1988) used adult abalones collected from the ocean. On the farm, hatchery managers select the fastest growing abalones to use as F1 broodstock (S. Ashlin, WCA farm hatchery manager, 2019, personal communication). This difference in the larval development rate could also be attributed to possible nutritional differences in the broodstock of the two studies and/or possibly due to the different larval culture techniques that were used. Larval rearing techniques have improved considerably since Genade et al. (1988) conducted their experiment, which might have led to faster growth in the present study.

No specific attention was given to the number of mortalities occurring during the planktonic stages of *Haliotis midae* development. We observed that patches of dead larvae (referred to in abalone hatcheries as "spotting") occurred more frequently between the developmental stage of pre-eye spot development and just before they were competent to settle. Genade et al. (1988) reported that no reliable data were obtained on larval (*H. midae*) mortality during the course of the experiments—larvae reared at 17.5°C and 20°C developed until they were competent to settle. Larvae in this experiment also developed until they were competent to settle in all treatments.

Once larvae are competent to settle, they have to move from being planktonic to benthic, and thus begin searching for a suitable substrate upon which to settle and metamorphose (Genade et al. 1988, Freeman 2001). The current study found a positive rate of larval settlement in all temperature treatments, except for the 14°C treatment. This indicates a strong relationship between temperature and settlement of *Haliotis midae* larvae. Larvae raised at 22°C had the highest percentage of settlement, whereas those at 14°C had the lowest (see Fig. 1). Similar results were reported for the bay barnacle *Amphibalanus improvisus* by Nosrolahi (2012), showing that the highest settlement occurred at 20°C with almost zero at 12°C. This was within the natural temperature range of *A. improvisus* between 0°C in winter up to 23°C during late summer (Nosrolahi 2012). Leighton (1972) reported that there was a higher percentage of settled *Haliotis sorenseni* larvae at warmer temperatures between 12°C and 20°C and zero settled larvae at 10°C, whereas larvae were still alive in all treatments after 15 days. The results by Leighton (1972) indicate that poor settlement of larvae do not mean mortality, which is the case in the present study with a poor settlement at 14°C. These results indicate that apart from chemical and physical inducers for settlement, temperature also plays a significant role in the settlement of *H. midae* larvae, and within the optimal temperature range for the species, warmer temperatures result in a better settlement.

After settlement when conditions are not suitable for metamorphosis, competent larvae attach to the substrate, crawl, and then swim off to the surface (Morse 1991, Moss & Tong 1992, Moss 1999). The current study's results show that at 14 and 16°C, larvae were still swimming at the end of the experiment after 10 days, with more swimming larvae at 14°C than at 16°C. Moss (1999) reported that cultured *Haliotis australis* larvae appear to settle but then swim off the surface when seawater temperature was between 10°C and 12°C, and this behavior lasted up to 8 days. Roberts and Lapworth (2001) reported that *Haliotis iris* larvae were competent to settle 7 days after fertilization but could still survive for 34 days without metamorphosis; however, only 4% of post-larvae survived after

completing metamorphosis 34 days later. As this experiment was terminated after 10 days, larvae at 14°C and 16°C may still have had a chance to metamorphosis sometime later, but the effects of delayed metamorphosis on *Haliotis midae* larvae need to be quantified to determine the dispersal window at lower temperatures.

When conditions are suitable, larvae cast off their swimming velum and change from being swimming larva into benthic juveniles undergoing the associated process of metamorphosis. Metamorphosis is an irreversible process and involves the mantle membrane separating from the larval shell and moving to the shell edge, after which juvenile shell growth commences (Leighton 2008). Larvae of *Haliotis midae* reared at 18°C, 20°C, and 22°C had all metamorphosed, and no larvae were swimming in the water column at the end of the experiment, whereas those at 14°C and 16°C had not yet metamorphosed and larvae were still swimming in the water column at approximately 10% of the initial density. Nosrolahi (2012) reported that when larvae of the barnacle *A. improvisus* were cultured at higher temperatures, it increased the metabolic rate to support a high growth rate. By contrast, for those maintained at low temperatures, more energy costs are associated with calcification (shell formation) in cold water. Therefore, they allocate more energy toward vital processes such as somatic mass maintenance at the expense of calcification. This might be the case in this study; abalone larvae at 14°C and 16°C appeared to be competent to settle but may have been portioning more energy toward maintaining their somatic mass instead of toward calcification of their shell.

Natural diatom films are commonly used to induce larval settlement in abalone hatcheries around the world (Roberts 2001). Biofilms developed from the natural diatoms in the incoming seawater were used in this study, and therefore, the species composition was not controlled and recorded. The percentage of settled larvae ranged between 2.56% ± 0.61% and 51.98% ± 23.95%, which is substantially higher than what is considered acceptable settlement (≈5%) by commercial hatcheries (S. Ashlin, WCA farm hatchery manager, 2019, personal communication); however, juveniles are only counted in the hatchery 3 months after settlement. A study by Daume et al. (1999) reported that the percentage settlement of *Haliotis laevigata* larvae on different species of cultured diatoms ranged between 15% and 45% after 15 days. Similarly, Li et al. (2006) reported that the larval settlement of *Haliotis diversicolor* was 25% on plastic sheets colonized by natural diatoms after 6 days. This suggests that although settlement can be highly variable, the percentage of settlement of *Haliotis midae* in this study was within the range of other abalone species.

The results from this study on modeling DD of larval development could also be used in abalone farm hatcheries. The DD model can be used to predict developmental stages for *Haliotis midae* larvae at different temperatures using data presented in this study. This information is crucial for effective control of production in the hatchery as it impacts the spawning schedule, diatom availability, nursery space, and logistics. The results are also relevant to abalone larval stock enhancement; seeding *H. midae* larvae at warmer temperatures will result in a higher settlement, which would reduce dispersal of larvae outside of the seeding area. Rearing *H. midae* larvae at temperatures between 18°C and 22°C might also result in faster growth for juveniles. This is because an increased temperature within an

optimal range of the species may increase the metabolic rate at a level that can still support an increase in growth (Pedroso 2017). This hypothesis is supported by Britz et al. (1997), who reported that the growth rate of juvenile *H. midae* increased across a 12°C–20°C temperature range. This will be advantageous for both hatcheries and stock enhancement programs because this temperature range (18°C and 22°C) corresponds to the sea temperature range experienced by *H. midae* within its natural environment.

In conclusion, temperature controls the planktonic development, settlement, and metamorphosis of *Haliotis midae* larvae. Moreover, raising *H. midae* larvae at 22°C increased the developmental rate and yielded a high settlement percentage. This result will benefit the abalones stock enhancement program—seeding larvae at warmer temperatures will increase the growth rate of abalone larvae and juveniles. It will reduce the potential of dispersing outside the seeding area and therefore reduce the probability of mortality by predation during the dispersal window. Last, the warmer temperature will increase the percentage of the settlement leading to

metamorphosis of *H. midae* larvae, thereby increasing the possibility of rebuilding the wild stock.

Further research is necessary to investigate the long-term effect of temperature on the development of recently settled juvenile abalones, and to assess the physiological effects of *Haliotis midae* larval development at temperatures above 22°C and below 14°C.

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LITERATURE CITED

- Bates, D., M. Maechler, B. Bolker & S. Walker. 2015. Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* 67:1–48.
- Britz, P. J., T. Hecht & S. Mangold. 1997. Effect of temperature on growth, feed consumption and nutritional indices of *Haliotis midae* fed a formulated diet. *Aquaculture* 152:191–203.
- Chezik, K. A., N. P. Lester & P. A. Venturelli. 2014. Fish growth and degree-days II: selecting a base temperature for an among-population study. *Can. J. Fish. Aquat. Sci.* 71:1303–1311.
- Daume, S., S. Brand-gardner & W. J. Woelkerling. 1999. Settlement of abalone larvae (*Haliotis laevigata* Donovan) in response to non-geniculate coralline red algae (Corallinales, Rhodophyta). *J. Exp. Mar. Biol. Ecol.* 234:125–143.
- De Waal, S. W. 2002. Factors influencing the ranching of the abalone species *Haliotis midae* along the namaqualand coast of South Africa. PhD, University of Cape Town. pp. 1–254. Available at: <http://hdl.handle.net/11427/10424>.
- De Waal, S. W. & P. Cook. 2001. Quantifying the physical and biological attributes of successful ocean seeding sites for farm-reared juvenile abalone (*Haliotis midae*). *J. Shellfish Res.* 20:857–861.
- Fox, J. & S. Weisberg. 2019. An R companion to applied regression, 3rd edition. Thousand Oaks, CA: Sage.
- Freeman, K. A. 2001. Aquaculture and related biological attributes of abalone species in Australia – a review. *Fish West Aust.* 128:1–55.
- Genade, A. B., A. L. Hirst & C. J. Smit. 1988. Observations on the spawning, development and rearing of the South African abalone *Haliotis midae* Linn. *S. Afr. J. Mar. Sci.* 6:3–12.
- Godfrey, B. P. 2003. The potential of abalone stock enhancement in the Eastern Cape province of South Africa. MSc, Rhodes University. pp. 1–170. Available at: <http://core.ac.uk>.
- Grubert, M. A. & A. J. Ritar. 2004. The effect of temperature on the embryonic and larval development of blacklip (*Haliotis rubra*) and greenlip (*H. laevigata*) abalone. *Invertebr. Reprod. Dev.* 45:197–203.
- Khoder, B., J. Moal & R. Robert. 2010. Impact of temperature on larval development and evolution of physiological indices in *Crassostrea gigas*. *Aquaculture* 309:286–289.
- Leighton, D. 1972. Laboratory observations on the early growth of the abalone, *Haliotis sorenseni*, and the effect of temperature on larval development and settling success. *Fish Bull.* 70:373–381.
- Leighton, P. 2008. Abalone hatchery manual. *Irish Sea Fish Board* 25:1–89.
- Li, H., W. Lin, G. Zhang, Z. Cai, G. Cai, Y. Chang & K. Xing. 2006. Enhancement of larval settlement and metamorphosis through biological and chemical cues in the abalone *Haliotis diversicolor* supertexta. *Aquaculture* 258:416–423.
- Lopez, L. M., P. A. Tyler & M. T. Viana. 1998. The effect of temperature and artificial diets on growth rates of juvenile *Haliotis tuberculata* (Linnaeus, 1758). *J. Shellfish Res.* 17:657–662.
- Malzahn, A. M., C. Clemmesen & H. Rosenthal. 2003. Temperature effects on growth and nucleic acids in laboratory-reared larval coregonid fish. *Mar. Ecol. Prog. Ser.* 259:285–293.
- McCormick, T. B., G. Navas, L. M. Buckley & C. Biggs. 2016. Effect of temperature, diet, light, and cultivation density on growth and survival of larval and juvenile white abalone *Haliotis sorenseni* (Bartsch, 1940). *J. Shellfish Res.* 35:981–992.
- Morse, A. N. C. 1991. How do planctonic larval know where to settle? *Am. Sci.* 79:154–167.
- Moss, G. A. 1999. Factors affecting settlement and early post-settlement survival of the New Zealand abalone *Haliotis australis*. *N. Z. J. Mar. Freshw. Res.* 33:271–278.
- Moss, G. A. & L. J. Tong. 1992. Techniques for enhancing larval settlement of the abalone, *Haliotis iris*, on artificial surfaces. *N. Z. J. Mar. Freshw. Res.* 26:75–79.
- Muller, L. 2003. Effect of illumination, shading and temperature on the settlement, survival and growth of juvenile abalone (*Haliotis midae*). MSc, University of Cape Town. pp. 1–100. Available at: <http://hdl.handle.net/11427/6973>.
- Nosrolahi, A. 2012. Stress ecology: interactive effect of temperature and salinity on early life stages of barnacle, *Amphibalanus improvisus*. PhD, Christ University. pp. 1–153. Available at: http://oceanrep.goemar.de/20110/1/Nasrolahi_Diss.pdf.
- Onitsuka, T., T. Kawamura, S. Ohashi, S. Lwanaga, T. Horii & Y. Watanabe. 2010. Effects of delayed metamorphosis and delayed post-settlement feeding on post-larval survival and growth of the abalone *Haliotis diversicolor*. *Aquaculture* 298:239–244.
- Pedroso, F. L. 2017. Effects of elevated temperature on the different life stages of tropical mollusk, donkey's ear abalone (*Haliotis asinina*). *Aquacult. Aquarium Conserv. Legis.* 10:1421–1427.
- R Core Team. 2020. R: a language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. Available at: <https://www.R-project.org/>.

- DAFF Report. 2016. Status of the South African Marine Fishery Resources.
- Roberts, R. D. 2001. A review of settlement cues for larval abalone (*Haliotis* spp.). *J. Shellfish Res.* 20:571–586.
- Roberts, R. D. & C. Lapworth. 2001. Effect of delayed metamorphosis on larval competence and post larval survival and growth, in the abalone *Haliotis iris* Gmelin. *J. Exp. Mar. Biol. Ecol.* 258:1–13.
- Samsing, F., F. Oppedal, S. Dalvin, I. Johnsen, T. Vågseth & T. Dempster. 2016. Salmon lice (*Lepeophtheirus salmonis*) development times, body size, and reproductive outputs follow universal models of temperature dependence. *Can. J. Fish. Aquat. Sci.* 73:1841–1851.
- Scharf, L., H. Braf, N. Ifrach, S. Rosenstein & A. Subach. 2015. The effects of temperature and diet during development, adulthood and mating on reproduction in the red flour beetle. *PLoS One* 10:1–15.
- Searle, T., R. D. Roberts & P. M. Lokman. 2006. Effects of temperature on growth of juvenile Blackfoot abalone, *Haliotis iris* Gmelin. *Aquacult. Res.* 37:1441–1449.
- Smit, A. J., M. Roberts, R. J. Anderson, F. Dufois, S. F. J. Dudley, T. G. Bormann, J. Olbers & J. J. Bolton. 2013. A coastal seawater temperature dataset for biogeographical studies: large biases between in situ and remotely-sensed data sets around the coast of South Africa. *PLoS One* 8:1–13.
- Visser-Roux, A. 2011. Reproduction of the South African abalone, *Haliotis midae*. PhD, Stellenbosch University. pp. 1–102. Available at: <http://scholar.sun.ac.za/handle/10019.1/18111>.



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The use of chemical and biological settlement cues in enhancing the larval settlement of abalone (*Haliotis midae*): Implications for hatcheries and ocean ranching

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Abstract

Reducing the time to settlement and metamorphosis of abalone larvae is critical for ensuring that abalone larvae settle within the seeding site for ocean ranching or to increase production in hatcheries. This study investigated the effect of biological (planktonic *Nitzschia* sp.) and chemical (potassium chloride) cues in inducing settlement and metamorphosis of abalone larvae *Haliotis midae* on diatom-coated plastic sheets. Larvae were exposed to different concentrations of KCl (10–20 mM), with settlement being highest at 10 mM in the first 20 h. Settlement of larvae exposed to a combined KCl and *Nitzschia* treatment for 24 h was highest, followed by larvae exposed to KCl for 12 h, while larvae exposed to KCl for 24 h, and both controls (12 and 24 h) had the lowest settlement. However, in both experiments, larval settlement in all treatments declined after 24 h of exposure, while that of the controls (no added settlement cues) increased and surpassed the other treatments after 24 h. Finally, the settlement was very low on uncoated sheets, compared to diatom-coated sheets, regardless of exposure to different combinations of KCl and *Nitzschia*. The exposure period's results should be interpreted with caution when drawing biological conclusions for field

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studies. This is due to the dramatic decrease in mean settlement post-exposure to the cue. Therefore, we hypothesize that exposure of *H. midae* larvae to 10 mM KCl and *Nitzschia* sp. will not enhance settlement in the ocean, as the inducers are primarily only effective at a KCl concentration level equal to 10 mM for 12 h. However, long-term exposure to KCl and *Nitzschia* over 24 h could be used in hatcheries to improve the settlement of *H. midae* larvae.

KEYWORDS

acute exposure, chronic exposure, inducer, KCl, metamorphosis, *Nitzschia*

1 | INTRODUCTION

The South African abalone (*Haliotis midae*) is the most commercially valuable species in the local aquaculture industry (DAFF, 2016). Its high value has driven illegal fishing resulting in the decline of the wild stock population, which has been well documented (de Waal, 2002; Godfrey, 2003; Raemaekers et al., 2011; Witte, 2017). Both stock enhancement and land-based abalone farming require a large number of hatchery-produced larvae (Li et al., 2006). Stock enhancement programs typically use land-based farmed juvenile abalone (de Waal, 2002; Godfrey, 2003); however, the production cost of raising juvenile abalone on a land-based abalone farm is high. Therefore, the seeding of abalone larvae has been considered a viable alternative. One of the significant constraints with seed production on abalone farms is poor larval settlement and metamorphosis, which results in low post-larval survival (Gapasin & Polohan, 2004). Li et al. (2006) reported that a maximum of 10% of larvae develop into metamorphosis, while the remaining larvae die, which is also more likely to happen in the wild under uncontrolled conditions. Therefore, reducing the time between larval release and settlement in the wild should reduce the overall larval mortality and dispersion outside the seeding area.

Many studies have focused on improving settlement and metamorphosis in commercially valuable abalone species from chemical cues such as γ -aminobutyric acid (GABA) (Gapasin & Polohan, 2004; Li et al., 2006; Moss & Tong, 1992; Stewart et al., 2008), potassium ions (Gapasin & Polohan, 2004; Li et al., 2006; Yool et al., 1986; Yu et al., 2010), bromomethane (Kang et al., 2004), and biological cues such as natural biofilms (Gordon et al., 2004) and conspecific mucus (Slattery, 1987).

Potassium chloride (KCl) is one of the most commonly used chemical cues and is an effective chemical settlement inducer that is also inexpensive and readily available. Literature investigating the effect of KCl has generally focused on improving settlement and metamorphosis within a controlled hatchery-like environment. For example, Bryan and Qian (1998) exposed competent *Haliotis diversicolor* larvae to a chemical cue for 96 h without water exchange. Likewise, Gapasin and Polohan (2004) exposed *Haliotis asinina* early veliger larvae (5–6 h post-hatch) to KCl until post larvae, 72 h post initial exposure, without any water exchange. Li et al. (2006) exposed *H. diversicolor* trochophore larvae to KCl until settlement and metamorphosis. In abalone hatcheries, water flow is turned off during settlement until no larvae are observed swimming in the water column, usually after ± 72 h (S. Ashlin, WCA hatchery manager, per. com). Although previous studies have emphasized the application to aquaculture, little is known about the viability of using chemical cues to improve the settlement and metamorphosis of competent farmed larvae released into the ocean.

This study aimed to investigate the settlement and metamorphosis of competent *H. midae* larvae exposed to KCl and the benthic diatom *Nitzschia*. This was to approximate the methodology used to enhance larval survival and

settlement if larval seeding in the ocean is shown to be viable. First, an optimal concentration of KCl to induce *H. midae* larval settlement was determined. Although the inducing effect of KCl has been tested on various abalone species, the responses of individual species are highly variable, even to the same inducing cue (Li et al., 2006). The optimal chemical concentration for each species should be known to maximize the settlement and metamorphosis. Next, using the optimal concentration of KCl, the effect of acute and chronic exposure of competent *H. midae* larvae to KCl and *Nitzschia* (free-swimming) before ocean seeding on the reef was investigated. This was achieved through laboratory experiments designed to simulate ocean seeding after exposure in captivity by comparing the larval settlement during and after exposure for different exposure durations. The following hypotheses were tested: (1) chronic exposure of *H. midae* larvae to settlement cues (KCl and *Nitzschia*) before seeding on the reef will increase settlement and metamorphosis compared to acute exposure to the cues; (2) settlement and substrate cue: exposure of abalone larvae to a combination of settlement cues and suitable substrate will result in more significant settlement and metamorphosis.

2 | MATERIALS AND METHODS

2.1 | Abalone spawning and larval rearing

Fertilized abalone (*H. midae*) eggs were collected within hours of deposition and hatched in 1 µm filtered seawater maintained at 17°C. Newly hatched out of the egg membrane larvae (trochophore stage) were transferred into a flow-through rearing system, with each aquarium receiving independent aeration and filtered (1 µm) UV-treated seawater. The photoperiod was set at 12:12 h. Ambient temperature was controlled with air conditioners and maintained at 18°C. Larvae were developed until they reached a stage where they could settle—6 days post-fertilization.

2.2 | Experimental system

Abalone larvae (*H. midae*) that are competent to settle were transported in 25 L buckets from Wild Coast Abalone (Pty) Ltd (WCA) farm to Kingfish Enterprises (Pty) Ltd farm, East London, South Africa. The competent stage of development was determined by the appearance of the third tubule on the cephalic tentacle, which indicates the ability to settle and crawl on the surface (Leighton, 2008). The experimental systems were designed as a water bath system, with each aquarium suspended in temperature-regulated water, maintaining the system's temperature. A flow-through system was used, with the water exiting via a banjo sieve covered with 80 µm mesh. A banjo sieve is a filter design that allows the water to exit the larval-rearing tank via a mesh while blocking the larvae from the tank (Leighton, 2008). The treatments were randomly allocated, with each 10 L aquarium (for experiment one) or 15 L aquarium (for experiments two and three) receiving independent aeration and water. Each water flow was set at 12 L h⁻¹, and a water chiller (Titan 2000, Aqua Medic) was used to maintain the temperature at 18°C. The temperature in each aquarium was monitored daily throughout the experiment (0.1°C variation). For each experiment, aquaria were stocked at two larvae.mL⁻¹ the standard stocking density used in the hatchery (S. Ashlin, Wild Coast Abalone farm-hatchery manager, pers. com).

2.3 | Diatom sheet production

Stock cultures of the benthic diatoms, *Amphora tenerima*, *Nitzschia laevis*, and *Navicula cf. vimnoid*, used in these experiments were supplied by Wild Coast Abalone Farm (Pty) Ltd, South Africa. The cultures were maintained at Kingfish Enterprises (Pty) Ltd, South Africa, in the temperature controlled (20 ± 1°C) laboratory in 1 µm filtered

seawater. Diatom cultures were fertilized using Cell-Hi F2F (Varicon Aqua Solution Ltd, England) and Sodium metasilicate (P E chemicals CC, South Africa) while the photoperiod was set at 24 hr light under fluorescent lighting.

Plastic sheets ($15 \times 10 \text{ cm} = 300 \text{ cm}^2$ total surface area) were placed in tanks containing a mixture of equal parts of each cultured diatom (33.3% each culture) (*Amphora* sp., *Nitzschia* sp., and *Navicula* sp.) in seawater. Aeration was provided, a 75% water exchange was performed twice a week, and fertilizer and more cultured diatoms were added to maintain good water quality. Diatoms were allowed to settle and proliferate for 3 weeks yielding a thin film on the surface of the sheets.

2.4 | The optimal concentration of KCl for *H. midae* larval settlement

Bryan and Qian (1998) suggested that the optimal concentration for potassium ions to induce larval settlement for *H. diversicolor* ranged between 10 and 20 mM. For this study, a control (with no KCl) and three KCl (Sigma–Aldrich, South Africa) concentrations (10, 15, and 20 mM) were used. A KCl stock solution was prepared using $1 \mu\text{m}$ filtered seawater and diluted for each treatment. Clean-filtered seawater was used in the control treatment, with three replicates per treatment. Water flow was switched off in all treatments (including the control) before adding the KCl. Two settlement sheets ($15 \times 10 \text{ cm} = 300 \text{ cm}^2$ total surface area) coated with diatoms were suspended in each aquarium to provide an adequate larval settlement for each treatment. Approximately 20,000 (2 larvae mL^{-1}) abalone larvae were added to each 10 L aquarium, and the larvae were exposed to the KCl for 6 h, after which the water flow was turned back on. During and after the exposure, settlement sheets were briefly removed from each aquarium and photographed at high magnification (Canon camera EOS 2000D). The number of larvae settled was counted on both sides of each sheet using DotDotGoose software, version 1.1.1 (Ersts, 2019). The experiment ran for 94 h, with counting occurring at increasing intervals. To determine the number of larvae still swimming in the water column, at the end of the experiment (94 h), a triplicate of 10 mL water samples was collected from each aquarium and counted under a dissecting microscope (Best Scope–3040 T, Lasec South Africa).

2.5 | The effect of the combination of settlement cues (chemical and biological) and exposure periods on larval settlement

This experiment was performed to determine the effect of the 12- and 24-h exposure period and the effect of different inducing cues on the larval settlement and metamorphosis. Three experimental treatments were used – larvae exposed to KCl for 12 h, 24 h exposure to KCl, 24 h exposure to a combination of KCl and planktonic *Nitzschia*, and two control treatments where water flow was stopped for 12 and 24 h, respectively but with no KCl or planktonic *Nitzschia* exposure. Three replicates were used for each treatment. The optimal concentration of KCl (10 mM) determined in the first experiment was used for all experimental treatments in this second experiment. Diatom cell concentration was calculated using a hemocytometer under a microscope, and a serial diluting of diatoms was done until the required number of cells was achieved. For the combination of KCl and *Nitzschia* exposed for 24 h, $120 \text{ cells mL}^{-1}$ of *Nitzschia* was added per aquarium. Approximately 30,000 larvae ($\pm 2 \text{ mL}^{-1}$) were added to each 15 L aquarium. Water flow was stopped for 12 or 24 h, exposing larvae to the treatment cues for the entire period. Water flow was then restored, and the abalone counting and recording method from the first experiment was followed. After counting the swimming larvae in the water column following the method described in the first experiment, water was carefully siphoned from each aquarium to avoid disturbing those larvae that might have settled on the surfaces of the aquarium. Settled abalone larvae were then removed from the sides of the aquarium by adding 1 M MgSO_4 , which acts as a muscle relaxant in abalone (White et al., 1996). Abalone were then removed by gently spraying them with seawater, concentrating them, and taking triplicate 10 mL samples from each aquarium which were counted under a dissecting microscope. After 94 h, the experimental settlement sheets were observed

at $\times 10$ magnification under the dissecting microscope (Best Scope-3040 T, Lasec South Africa) to determine the number of metamorphosed larvae on each sheet. Metamorphosed larvae were identified by shell growth (Leighton, 2008).

2.6 | Larval settlement in response to KCl and planktonic *Nitzschia* sp. after short exposure

This experiment determined the larvae's settlement response and metamorphosis after a brief exposure to the settlement cues. Five treatments were tested (a) KCl only, (b) planktonic *Nitzschia* only, (c) a combination of KCl and planktonic *Nitzschia*, (d) a control (with no KCl or *Nitzschia*), and (e) a reference control with the treatment that achieved the highest settlement in experiment two (24 h exposure to a combination of 10 mM KCl and planktonic *Nitzschia*). Again, the optimal concentration of KCl from the first experiment was used for all experimental treatments in the third experiment. All treatments were set up similarly as described in the second experiment, with three replicates for each treatment. Approximately 30,000 larvae were added to each 10 L aquarium (± 3 larvae mL^{-1}) and exposed to the respective treatment for 60 min. After 60 min, the settlement inducers were removed by gently collecting the larvae from each aquarium on a 60 μm screen. The screen was carefully removed from the bucket, and the larvae were removed by rinsing the screen into the 15 L aquarium with the flow-through system to assess post-exposure settlement. The reference and experimental treatment were set up in a 15 L aquarium. Following the method described in the second experiment, settlement sheets were counted pre- and post-turning off water flow, except in the treatment for the third experiment, two settlement sheets (one sheet coated with diatoms and the other uncoated) were added post-flushing the settlement cues. The immediate settlement was assessed after 2 h post-exposure at 6 h intervals for 24 h and then at 12 h intervals until 74 h. Metamorphosis was assessed following the same method as described in the second experiment.

2.7 | Statistical analysis

For all three experiments, generalized linear models were used to relate the number of larvae counted on the sheets to the duration of the experiment in hours as a continuous variable, the treatment as a factor variable, and the interaction effect between hours and treatment (R statistical software, R Core Team, 2021). This model gives separate intercepts and slopes for each treatment; therefore, the estimated change in settlement over time could be plotted for each treatment in an experiment. The Poisson distribution was used to model larval count data; therefore, the intercept and slope estimates were "back-transformed" to the original scale from the log scale. This means that for each treatment, the line equation was given by the power function: $\text{Larvae} \sim \text{Intercept}(\text{Slope}^{\text{hours}})$. Back-transformed slope estimates below 1 indicate negative relationships, and above 1 indicate positive relationships, and non-significant relationships have slope estimates with 95% confidence intervals that overlap 1. Further, the model output of each experiment was expressed as an analysis of covariance (ANCOVA) table with the likelihood ratio chi-square (χ^2) test statistic, and the p -value < 0.05 regarded as significant (R package 'car') (Fox & Weisberg, 2019). Model diagnostic plots of residuals vs. fitted values indicate mostly random residual values except for experiment 2, where there is an increased residual variation at higher fitted values. However, for all experiments, Poisson models fit better to the data compared to other options that were checked in preliminary tests, i.e., count data with a negative binomial distribution, percentage settlement with a binomial distribution, and an arc-sine transformed percentage settlement with a Gaussian distribution.

The number of metamorphosed larvae is a proportion of the number of settled larvae. Therefore, we use a generalized linear model with a binomial distribution family to analyze the variation in metamorphoses across treatments for experiments 2 and 3. In this model, the response variable is a binomial variable that combines the number of successes (metamorphosed larvae) and the number of failures (larvae that did not metamorphose), which allows the model to consider the total number of settled larvae in each treatment. To avoid pseudoreplication, the number of

larvae counted on both sheets were pooled from each treatment replicate. An exploration of the data suggests that the chances for metamorphosis are generally very high, as nearly all larvae had metamorphosed in most replicates. Therefore, a complementary log–log link was used in the model, which is appropriate when the probability of an event is very high or very low. The results for each experiment were expressed as an analysis of variance, and a Tukey Honestly Significant Difference (HSD) post hoc test was performed to reveal pairwise differences between treatments (R function 'glht' from R package 'multcomp', Hothorn et al., 2008).

3 | RESULTS

3.1 | The optimal concentration of KCl to induce *H. midae* larval settlement

Larval settlement differed significantly among the concentrations (different intercepts), and larval settlement also changed significantly over time at different rates (different slopes) in all concentrations (Tables 1 and 2). Initially, during the 6 h that larvae were exposed to KCl, the larval settlement was highest in the 10 mM KCl treatment and lowest in the 20 mM KCl treatment (Figure 1 and the intercepts in Table 2). However, larval settlement decreased over time if exposed to 10 mM KCl, while it increased over time in the other treatments, with the steepest increase occurring in the control treatment (Figure 1, Table 2). Consequently, after 16 h (Figure 1), larval settlement in control was higher ($5865 \pm 2302\text{SD}$, averaged over all values recorded between 23 and 94 h) compared to all other treatments (10 mM = $3963 \pm 2302\text{SD}$; 15 mM = $2010 \pm 951\text{SD}$; 20 mM = $2100 \pm 1069\text{SD}$). No larvae were found swimming in the water column at the end of the 94 h experiment.

3.2 | The effect of the combination of settlement cues (chemical and biological) and exposure periods on larval settlement

Larval settlement differed significantly among different exposure times, and larval settlement also changed significantly over time, with the rate of change differing among different exposure times in all

TABLE 1 Analysis of covariance output for all three experiments. The χ^2 test statistic is indicated for each term, together with the significance indicated symbolically.

Experiment	Hours	Treatments	Interaction
1. Different concentrations	12.152****	60.689*****	10.929****
2. Different exposure periods	1160.5****	33040.4****	21025.4****
3. Different cues	2565.308****	27027.1****	187.7****

Note: Symbol: **** <0.0001.

TABLE 2 Estimated slopes and intercepts for each KCl concentration, with 95% confidence intervals in brackets, that had been back-transformed from the log to the original scale.

Concentration	Intercept	Slope ^a
0	2643.569 (2618.744; 2668.63)	1.0131 (1.0129; 1.0133)
10	4521.506 (4464.846; 4578.884)	0.9991 (0.9989; 0.9994)
15	1712.026 (1684.935; 1739.552)	1.0046 (1.0042; 1.0049)
20	1398.709 (1375.438; 1422.374)	1.0069 (1.0066; 1.0073)

^aSlope estimates >1 represent positive relationships, and slopes <1 represent negative relationships.

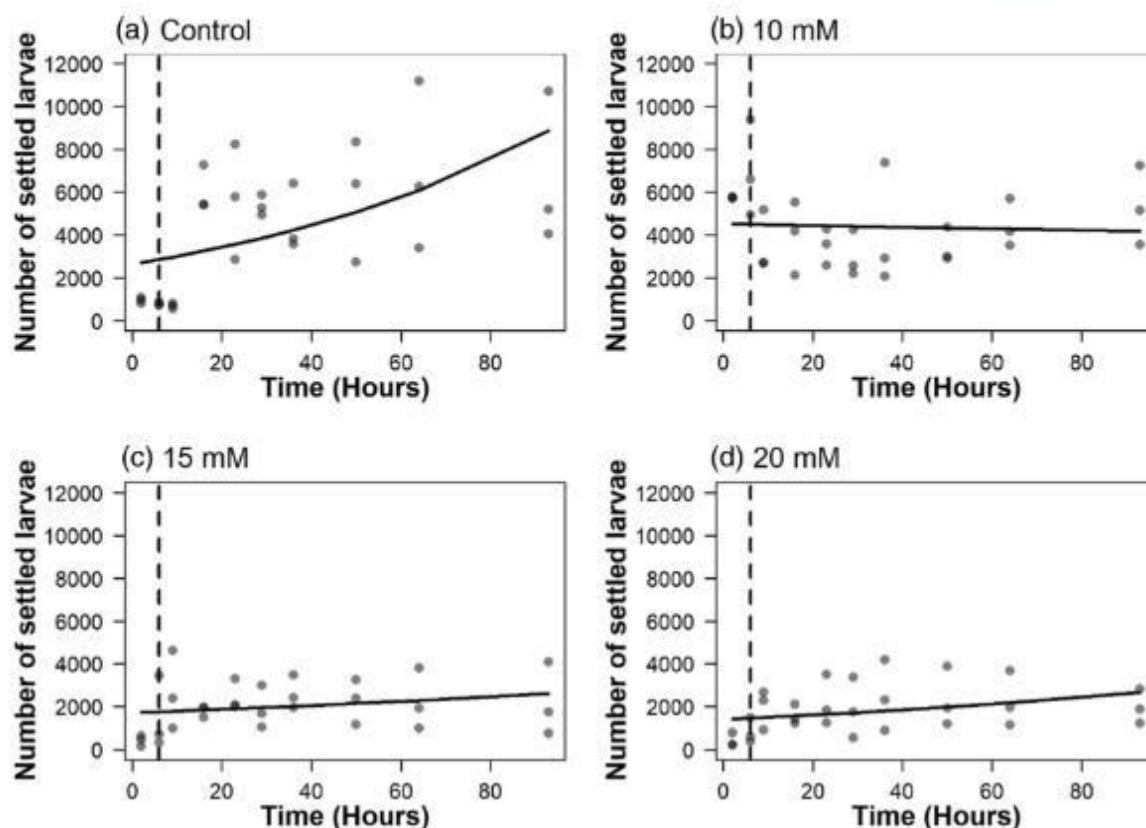


FIGURE 1 Number of settled abalone (*Haliotis midae*) larvae during and post-exposure to three different concentrations of potassium chloride (10, 15, and 20 mM).

TABLE 3 Estimated slopes and intercepts for each treatment, with 95% confidence intervals in brackets, that had been back-transformed from the log to the original scale.

Exposure	Intercept	Slope ^a
12 h control	323.754 (316.705; 330.960)	1.0239 (1.0236; 1.0243)
24 h control	374.437 (362.823; 386.422)	1.0089 (1.0084; 1.0095)
12 h KCl	1722.560 (1678.634; 1767.635)	0.9925 (0.9920; 0.9930)
24 h KCl	1018.593 (991.907; 1045.997)	1.0023 (1.0018; 1.0028)
24 h KCl and <i>Nitzschia</i>	2022.009 (1972.395; 2072.872)	0.9947 (0.9943; 0.9952)

^aFor a power function slope, values >1 represent positive relationships and slopes <1 represent negative relationships.

concentrations (different intercepts and slopes, Tables 1 and 3). Initially, the larval settlement was highest in the treatments where larvae were exposed to a combination of KCl and *Nitzschia* for 24 h and where larvae were exposed to KCl for 12 h (see intercepts in Table 3). In comparison, both control treatments had significantly lower initial larval settlement (see intercepts in Table 3). Therefore, the combination of KCl and *Nitzschia* exposure for 24 h was used as the reference control in the third experiment. However, larval settlement in the combined 24-h KCl and *Nitzschia* treatment and the 12 h KCl treatment declined over time, while larval settlement increased over time in the 24-h KCl treatment and controls (Figure 2 and slopes in Table 3). Consequently, after 24 h (Figure 2), larval settlement in the 12 h control was highest (1490 ± 820 SD averaged over all values recorded between 27 and 94 h), followed by the 24 h KCl and

Nitzschia combination treatment ($1151 \pm 483\text{SD}$), the 24 h KCl treatment ($1070 \pm 564\text{SD}$), the 12 h KCl treatment ($1023 \pm 443\text{SD}$), and the 24 h control ($584 \pm 322\text{SD}$).

At the end of the experiment (94 h), there were still 0.07 ± 0.07 larvae mL^{-1} larvae swimming in the 10 mM KCl 12 h and 0.13 ± 0.07 larvae mL^{-1} in 10 mM KCl 24 h treatment. Most larvae from all treatments had also metamorphosed, but the proportion of metamorphosed larvae differed significantly among treatments (ANCOVA: $\chi^2 = 2761.7$, $df = 4$, $p < 0.0001$). The Tukey HSD pairwise test shows that all treatments have a significantly higher proportion of metamorphosis than the two controls (Table 4). Pairwise differences were not significant

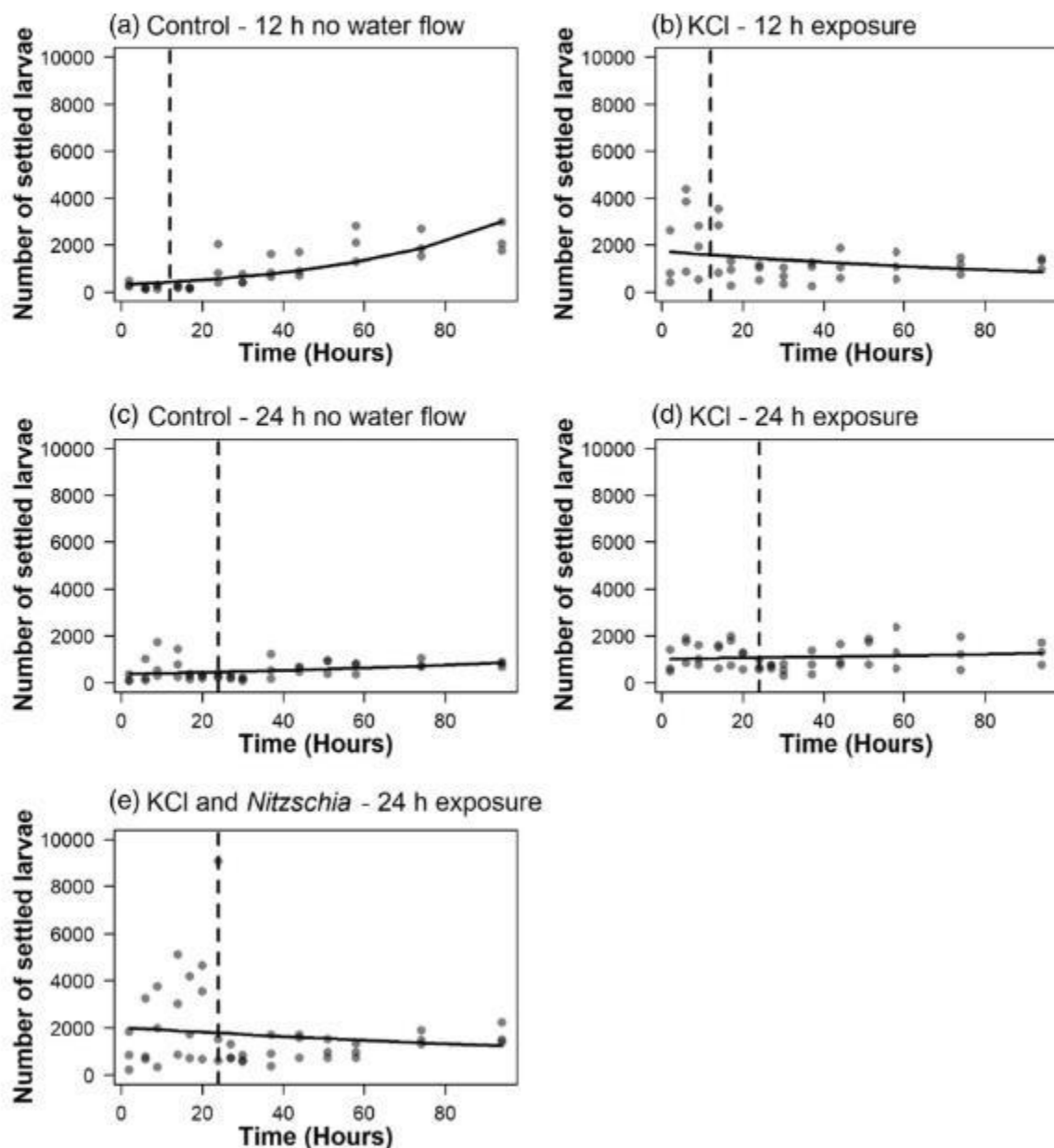


FIGURE 2 Number of settled abalone (*Haliotis midae*) larvae during and post-exposure for 12 h to 10 mM KCl, 24 h to KCl, and exposure to a combination of 10 mM KCl and *Nitzschia* for 24 h with control treatment for each exposure time. The vertical dashed line indicates exposure period for each treatment and no water flows for each control treatment.

between the KCl 12 h treatment and the Diatoms and KCl 24 h treatment, and the KCl 24 h treatment and the KCl 12 h treatment, while the significant difference between the KCl 24 h treatment and the Diatoms and KCl treatment is very weak. However, it is important to note that significantly lower metamorphosis in the controls may be due to three cases where low metamorphosis occurred on only one of the two sheets in the tank – one replicate of the 12 h control and two replicates of the 24 h control.

3.3 | Larval settlement in response to KCl and free-swimming *Nitzschia* sp. after short exposure

The larval settlement was negligible on blank sheets in all treatments and did not change over time (averaged over all samples $4 \pm 4SD$, Tables 1 and 5). On diatom-coated sheets, larval settlement differed significantly among the different treatments (Tables 1 and 5). Initially, the larval settlement was higher in a combined KCl and *Nitzschia* treatment

TABLE 4 Pairwise comparison of metamorphosis variation across settlement cue treatments and the controls.

Pairwise comparison	Estimate	SE	z value	p
Control 24 h, Control 12 h	−0.745	0.031	−24.077	<0.001
KCl and <i>Nitzschia</i> 24 h, Control 12 h	0.757	0.034	22.248	<0.001
KCl 12 h, Control 12 h	0.742	0.038	19.492	<0.001
KCL 24 h, Control 12 h	0.640	0.034	18.681	<0.001
KCl and <i>Nitzschia</i> 24 h, Control 24 h	1.502	0.040	37.211	<0.001
KCl 12 h, Control 24 h	1.487	0.044	33.938	<0.001
KCL 24 h, Control 24 h	1.385	0.041	34.147	<0.001
KCl 12 h, KCl and <i>Nitzschia</i> 24 h	−0.016	0.046	−0.343	0.997
KCL 24 h, KCl and <i>Nitzschia</i> 24 h	−0.117	0.043	−2.729	0.048
KCL 24 h, KCl 12 h	−0.101	0.046	−2.195	0.175

TABLE 5 Estimated slopes and intercepts for each treatment, with 95% confidence intervals in brackets, that had been back-transformed from the log to the original scale.

Treatment		Intercept	Slope ^a
Cue	Substrate		
Control	Blank	4.698 (3.445; 6.406)	0.9949 (0.9867; 1.0031)
	Diatoms	69.877 (50.879; 95.969)	1.0170 (1.0086; 1.026)
Reference control	Diatoms	101.125 (73.778; 138.608)	1.0153 (1.0068; 1.0237)
	Diatoms	103.588 (75.539; 142.053)	1.0087 (1.0003; 1.0172)
KCl	Blank	5.276 (3.442; 8.088)	0.9945 (0.9833; 1.0059)
	Diatoms	157.316 (114.939; 215.316)	1.0095 (1.0012; 1.0180)
<i>Nitzschia</i>	Blank	3.699 (2.356; 5.806)	1.0029 (0.9916; 1.0144)
	Diatoms	121.986 (89.065; 167.075)	1.0139 (1.0056; 1.0224)
KCl and <i>Nitzschia</i>	Blank	3.976 (2.538; 6.228)	0.9996 (0.9881; 1.0112)
	Diatoms	137.868 (100.693; 188.768)	1.0115 (1.0031; 1.0199)

^aFor a power function, slope values >1 represent positive relationships and slopes <1 represent negative relationships. If 95% confidence intervals of a slope estimate overlap 1, the relationship is insignificant.

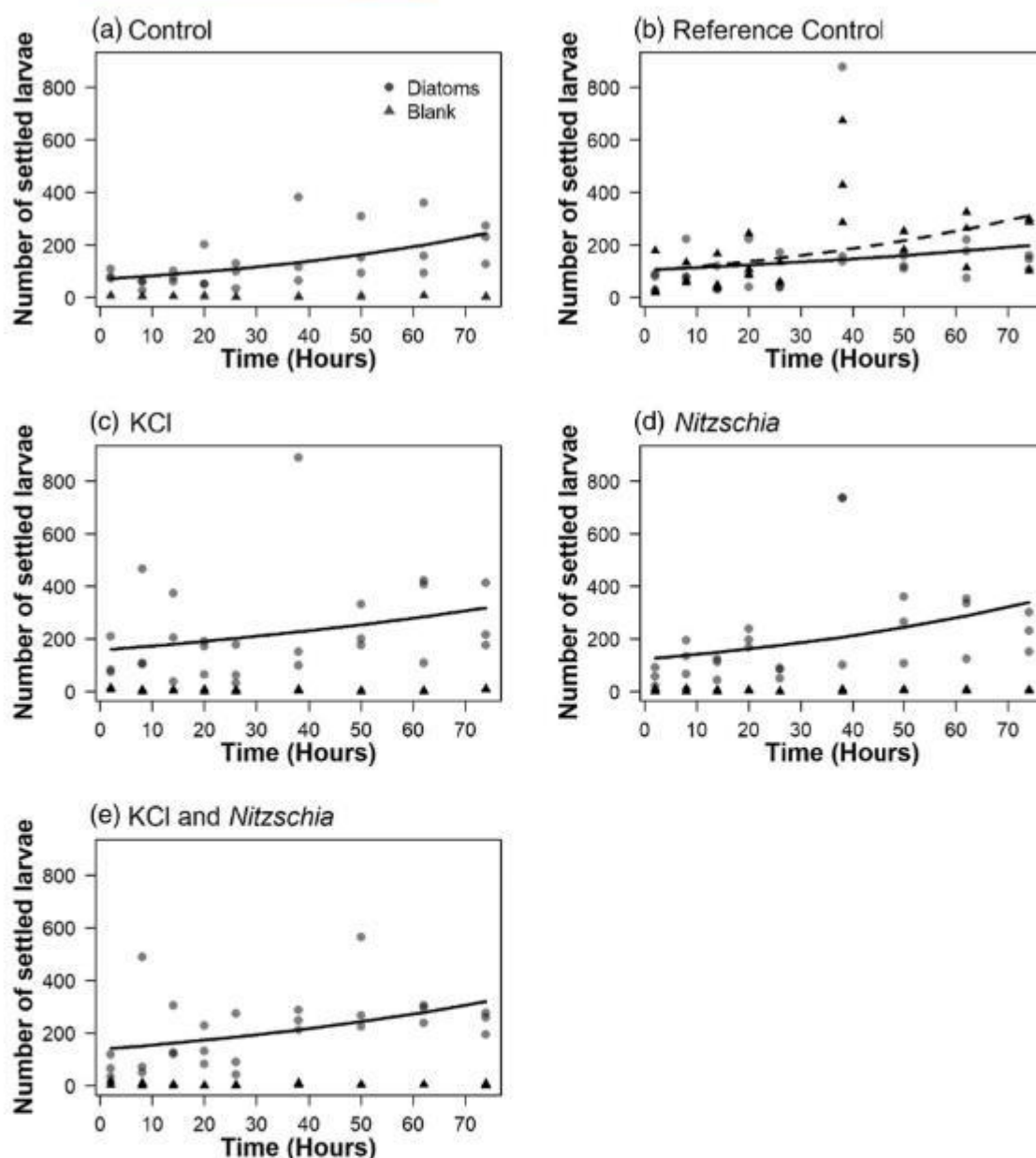


FIGURE 3 Number of settled abalone (*Haliotis midae*) larval after 60 min exposure to three settlement cues (10 mM KCl, *Nitzschia* sp, a combination of 10 mM KCl and *Nitzschia*) and a control (no *Nitzschia* or KCl) and reference treatment (10 mM KCl and *Nitzschia* exposed for 24 h) on diatoms coated sheets.

and KCl-only treatment, while the control treatment had the lowest larval settlement, but these differences were not as pronounced as in the other experiments (see, e.g., the intercepts in Table 5). The settlement on diatom-coated sheets increased over time in all treatments and controls (Figure 3 and the slope estimates in Table 5).

Larvae settled on the aquarium surface were calculated at the end of the experiment, and there was no difference (per.m^{-2}) between treatments. More larvae settled on the aquarium's surface than on the settlement sheet, either coated or uncoated. At the end of the experiment, the percentage of larvae swimming in the water column was not different between treatments. None of the few larvae that had settled on the blank sheets had metamorphosed; therefore, the statistical test was done only on the diatom-coated sheets. Although almost all larvae

TABLE 6 Pairwise comparison of metamorphosis variation across settlement cues on diatom-coated sheet treatments and the controls.

Pairwise comparison	Estimate	SE	z value	p
KCl, Control	-0.018	0.122	-0.151	1.000
Diatom & KCl, Control	-0.182	0.115	-1.589	0.499
Diatoms only, Control	-0.199	0.115	-1.727	0.412
Control, Reference control	-0.270	0.106	-2.561	0.076
KCl, Reference control	-0.252	0.095	-2.665	0.058
Diatom & KCl, Reference control	-0.088	0.086	-1.032	0.838
Diatoms only, Reference control	-0.071	0.086	-0.824	0.922
Diatoms & KCl, KCl	-0.164	0.105	-1.565	0.514
Diatoms only, KCl	-0.181	0.105	-1.716	0.419
Diatoms only, Diatoms & KCl	-0.017	0.097	-0.175	1.000

(>95.8% in all replicates) had metamorphosed on the diatom-coated sheets, a weak significant difference in metamorphosis was found among different treatments (ANCOVA: $\chi^2 = 11.6$, $df = 4$, $p = 0.02$). However, a Tukey HSD test found no significant pairwise differences between treatments or controls (all p values >0.057) (Table 6). Therefore, the significant ANCOVA p -value is not biologically meaningful and could possibly be a type I error.

4 | DISCUSSION

In the natural environment, competent larvae of marine gastropods tend to swim downward instinctively to explore the substrate by randomly testing the contacted surface. If the substrate is suitable, they will attach; otherwise, they will swim off in search of a suitable substrate for their future development and growing needs (Li et al., 2006). Larval settlement is highly influenced by chemical cues, the characteristic of a suitable environment for newly recruited individuals (Huggett et al., 2005). Moss and Tong (1992) reported that inducers such as synthetic chemicals (KCl and GABA) arrest the movement of the cilia of the veliger, forcing the abalone larva to move downwards. Chemical cues such as potassium chloride, dopamine, and glutamine can down-regulate larval swimming and induce early attachment and metamorphosis of *Haliotis iris* (Alfaro et al., 2014). Although the mechanism by which KCl induces settlement of abalone larvae is poorly understood, Baloun and Morse (1984) reported that an increase in the concentration of K^+ in a defined seawater medium induces settlement in larvae *Haliotis rufescens*. However, the first experiment in the current study showed that exposure to 10 mM KCl induced three times higher larval settlements than the higher concentrations (15 and 20 mM), suggesting that there is an optimal concentration of KCl to induce settlement. Li et al. (2006) reported a similar trend that 10 mM KCl induced settlement of *H. diversicolor*, and high concentration of KCl (20 mM) were toxic to the larvae resulting in a high level of mortality. Like Li et al. (2006), high concentrations of KCl might have been toxic to the larvae and hindered settlement in the current study. In contrast, Bryan and Qian (1998) showed that the percentages of settled *H. diversicolor* larvae was not different between 10 and 20 mM of KCl. The different response from Bryan and Qian (1998) study suggests that the same species might respond differently to chemical cues.

Continuous exposure of *H. rufescens* larvae to 10 mM K^+ increased the settlement (Yool et al., 1986). It was hypothesized that more prolonged exposure of *H. midae* larvae to settlement cues (KCl and *Nitzschia*) would maximize settlement and metamorphosis compared to larvae subjected to shorter exposure. However, in the second experiment, exposure of larvae to KCl for 12 h displayed higher overall settlement compared to the overall number of settled larvae exposed to KCl for 24 h and the control treatments. The difference between 12 h KCl and 24 h KCl

larvae exposure may be due to the difference in water quality between the treatments. Larvae (*Haliotis laevis*) are sensitive to poor water quality (Burke et al., 2001). Poor water quality stresses the abalone and slows its growth; in the worst case, water quality can deteriorate to the extent of causing mortalities (Burke et al., 2001). Therefore, stopping water flow for extended periods might have negatively affected the water quality to a point where it affected the settlement. However, prolonged exposure (24 h) to the combination of KCl and *Nitzschia* resulted in the highest larval settlement compared to all other treatments. Microalgae use carbon dioxide (produced by larvae through respiration) during photosynthesis, where oxygen is produced, and it absorbs ammonium and nitrate in the water during assimilation (Abdel-Rouf, 2012). The planktonic microalgae (*Nitzschia*) might have helped maintain good water quality in this treatment, resulting in the highest settlement. The settlement almost doubled in the combination of KCl and *Nitzschia* exposed for 24 h treatment compared to KCl exposed for 12 h during the exposure. One possible reason is that after 24 h post-exposure, *H. midae* larvae were more developed, which might have contributed to the settlement. Barlow (1990) noticed rapid settlement of *H. rufescens* in response to exposure to GABA in older larvae. Moss (1999) also reported that settlement was more significant for older larvae when compared with younger larvae. Another possibility is that the combination of two settlement cues, KCl and *Nitzschia*, is more likely to result in a higher settlement when compared to a single cue. Potassium chloride is known to down-regulate abalone swimming and force the larvae to settle (Alfaro et al., 2014; Moss & Tong, 1992), while *Nitzschia* releases aspartic acid, which strongly induces the attachment of swimming abalone larvae (Gordon et al., 2004). Similar results were reported by Li et al. (2006), showing that combining 10 mM KCl and natural diatoms could increase settlement by 48% compared to 28% for the control (natural diatoms) treatment.

Post-exposure settlement in the second experiment treatments showed a significant reduction in the mean number of settled larvae (Figure 2), as was observed in the first experiment (Figure 1). Barlow (1990) reported that at low concentrations, GABA does not paralyze cilia which cause settlement in larvae. Assuming a similar response occurs in larvae exposed to low concentrations of KCl, in this experiment, the water-flow post-exposure period diluted the concentration of KCl in the water, and larvae that had not metamorphosed could swim off the substrate. These results are supported by Morse (1991), who stated that it took up to 20 h for larval *H. rufescens* to shed its velum (the fringe of ciliated cells surrounding its head that allows it to swim). Similarly, Bryan and Qian (1998) reported that it takes up to 48 h for larval *H. diversicolor* to start metamorphosis after initial settlement. Therefore, it is likely that *H. midae* larvae had not yet shed their velum and, therefore, not metamorphosed by the end of the 6-, 12-, and 24-h exposure periods enabling them to swim off the settlement sheet when KCl concentration was reduced by flushing. The mean number of settled larvae in treatments with KCl was less than the control treatment at the end of the experiment, indicating that not all larvae in the KCl treatment reattach to the settlement sheet after the initial settlement.

In the first and second experiments, the settlement of larvae in the control treatments increased over time and could sometimes even surpass settlement compared to other treatments post-exposure. Barlow (1990) reported that unstimulated larvae spend less time swimming as they age. These results suggest that prolonged exposure (24 h) of *H. midae* larvae to either chemical cues (KCl) or a combination of chemical cues and biological (*Nitzschia*) before release may not increase settlement in the ocean as the inducers are primarily effective when KCl concentration levels are 10 mM. It is also possible that the abalone larvae might settle on the surface of the bucket before seeding during the hours of exposure.

Results from the third experiment showed that settled larvae were initially higher in the reference control treatment (combination of KCl and *Nitzschia* exposed for 24 h) compared to the diatom-coated sheets and the short exposure control. However, the short-exposure control treatment had a higher rate of settlement over time (greater slope value) on the diatom-coated sheets compared to the reference control (Table 5). Based on these results, the exposure period hypothesis was therefore rejected.

Settlement is affected by several factors, including biological cues associated with the substrate, which are considered the primary stimuli for initiating larval settlement in many marine mollusks (Li et al., 2006). Larvae must contact the substrate to be exposed to a biological cue that triggers settlement (Slattery, 1987). The larval settlement

differed between the treatments of diatom-coated settlement sheets. Daume et al. (1999) reported that diatoms induce settlement for abalone larvae *Haliotis rubra*, which is supported by Li et al. (2006), who showed that diatom films (with both mixed diatoms and bacteria) had better larval settlement than those exposed to uncoated sheets. Although the short exposure treatments were statistically different from the control, the treatments almost appear similar in a graph, suggesting that the difference was not big enough to have practical application for field-based studies or the hatchery. In addition, the settlement was not different between treatments and the control on uncoated sheets.

Specific environmental stimuli are required to initiate the metamorphosis of many marine invertebrate larvae (Yool et al., 1986). Once larvae find a suitable substrate to settle, metamorphosis follows shortly after, an irreversible commitment to the benthic mode of life (Morse, 1991). The mean number of larvae reaching metamorphosis was greater in the treatments compared with the controls for experiment 2 (Tables 4 and 6), while no meaningful difference was found between the treatments and the controls for experiment 3. For all replicates in experiment 3, more than 95% of settled larvae had metamorphosed. However, it is essential to note that for the second experiment, the lower proportions of metamorphosis in the controls are due to low metamorphosis (<17% metamorphosed) on three specific sheets – one 12 h control replicate and two 24 h control replicates. In each case, the rate of metamorphosis was high on the other sheet in the pair. On all other sheets, more than 95% of the larvae had metamorphosed in the controls and treatments, similar to experiment 3. The poor metamorphosis may be due to different layers (thin or thick) of diatoms, which affect settlement (S. Ashlin Wild Coast Abalone farm-hatchery manager, pers.com). Gapasin and Polohan (2004); Kang et al. (2004); Li et al. (2006) reported that after continuous exposure of larvae to KCl, the metamorphosis was induced. The process of metamorphosis begins 48 h post-settlement (Bryan & Qian, 1998; Morse, 1991). In the second and third experiments, larvae were exposed to the chemical (KCl) and the biological (*Nitzschia*) inducers for 12, 24, and 1 h before being flushed out. Therefore, metamorphosis in these experiments would appear to have not been influenced by either KCl or planktonic *Nitzschia*; instead, the biofilm coating on the settlement sheets may have induced larval metamorphosis. Gordon et al. (2004) reported that layers of diatoms such as *Nitzschia*, *Navicula*, and *Amphora* improved the attachment and settlement of abalone larvae *Haliotis discus hannai*. Maximizing larval metamorphosis is essential for the success of the abalone hatcheries, ranching, and stock enhancement programs; these findings can therefore guide abalone farmers and stock enhancement programs managers in the selection and use of appropriate settlement cues that will increase larval settlement and metamorphosis.

Larvae are eventually forced to settle once their endogenous yolk reserve is exhausted, even if they have not yet been exposed to a settlement inducer (Gapasin & Polohan, 2004). In the third experiment, the small percentage of larvae (less than 1%) that settled on the uncoated sheet had not yet metamorphosed after 74 h. Li et al. (2006) reported similar results, who showed that 5% of *H. diversicolor* larvae that settled on the uncoated surface (with no settlement cues) did not metamorphose. Gapasin and Polohan (2004) reported that in the absence of settlement cues, exploring larvae will ultimately settle without inductive cues once the endogenous yolk reserve is exhausted. Hence, in this study, some larvae were observed settled on the surface of the aquarium, probably because of the absence of cues to induce the settlement of uncoated sheets.

At the end of the second experiment (94 h), some larvae were still swimming in both KCl 12- and 24-h exposure treatments. Similarly, in the third experiment, some larvae were still swimming after 72 h in all treatments. Reasons for this could be the following. (a) The larvae quality varied among studies. The current study was limited to the broodstock supplied at the facility, and the confounding effect related to larval batch quality was regrettably unavoidable. (b) Slight differences in the diatom layer on the settlement sheets may have existed. Different layers (thin or thick layers) of diatoms can affect the settlement of abalone larvae (S. Ashlin, Wild Coast Abalone Farm-hatchery manager, pers.com). (c) In experiment two, *H. midae* larvae were 9 days old at the end of the experiment compared to the eight-day-old larvae in experiment three. Moss and Tong (1992) reported that the developmental stage of larval affected the settlement of *H. iris*, with higher settlement occurring for older larvae.

The inducer effect of KCl on the settlement and metamorphosis of a variety of marine invertebrates larvae has been widely acknowledged (Bryan & Qian, 1998; Gapasin & Polohan, 2004; Kang et al., 2004; Li et al., 2006; Yool et al., 1986; Yu et al., 2010). However, the results from this study differ from other studies. One possible reason for

the inconsistencies could be that *Haliotis* spp. behave differently, in that what is effective for one species may or may not necessarily trigger the same response for others (Yu et al., 2010). Another possible reason is that the current study used a commercial hatchery larval stocking density (2 larvae mL⁻¹), a typical density used in South African *H. midae* hatcheries (S. Ashlin, Wild Coast Abalone farm-hatchery manager, per. com). Other studies have, however, used 0.2 larvae mL⁻¹ (Bryan & Qian, 1998; Gapasin & Polohan, 2004; Kang et al., 2004; Li et al., 2006; Yu et al., 2010). Liu et al. (2006) reported that increased rearing density increases the possibility of collision among individuals causing an abrupt cessation of the beating of the preoral cilia, followed by rapid retraction of the velum. Abalone larvae swim with an extended velum which, if retracted, results in the larvae being unable to swim, therefore resulting in them sinking to the bottom of the aquarium (Alfaro et al., 2014). This reduces the chances of the larvae settling on the suspended settlement sheets. Finally, inconsistencies between the current study's findings and those of previous studies may result from slight differences in experimental design. In the studies of Bryan and Qian (1998); Gapasin and Polohan (2004); Li et al. (2006), abalone larvae were exposed to the chemical inducer for ± 5 days with a partial dilution of water once a day in some experiments or none at all in others. In this study, *H. midae* larvae were exposed to the chemical and biological inducer for varying periods (1, 6, 12, and 24 h), and water flow was resumed post-exposure. Morse (1991) reported that abalone larvae must contact the inducer substrate. In this experiment, flushing out of the KCl post-exposure significantly affected larval settlement, which may be why the settlement was reduced post-exposure, and the results are different from others with continuous exposure.

In conclusion, longer exposure (+12 h) of *H. midae* larvae to 10 mM KCl and *Nitzschia* enhanced settlement. This suggests that continuous exposure of larvae to an inducer until metamorphosis, which could be achieved in a hatchery but not in the ocean, will increase settlement. These results also showed a dramatic decrease in settlement of *H. midae* larvae post +12 h exposure, while 60 min exposure to KCl and *Nitzschia* did not result in a settlement difference that was big enough to have practical application in the field-based studies or the hatchery. These results suggest that exposure of *H. midae* larvae to biological (*Nitzschia*) and chemical cues (10 mM KCl) for 24 h or less before release may not increase settlement in the ocean. The inducers are primarily effective when KCl concentration levels equal 10 mM. Therefore, these results cannot draw a biological conclusion for field studies due to poor settlement when the biological cues are not highly concentrated (120 cells mL⁻¹), and KCl levels in seawater are typically below 10 mM (Todd et al., 1991), and it does not improve settlement without adding extra KCl in the seawater.

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CONFLICT OF INTEREST STATEMENT

The following authors: Ziyanda Mzozo, Sanet Hugo, and Niall Vine, have no conflicts of interest to declare. All co-authors have seen and agree with the manuscript's contents, and there is no financial interest to report. We certify that the submission is original work and is not under review at any other publication.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article.

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REFERENCES

- Abdel-Rouf, N. (2012). Microalgae and wastewater treatment. *Saudi Journal of Biological Sciences*, 19(3), 257–275.
- Alfaro, A., Young, T., & Bowden, K. (2014). Neurophysiological control of swimming behaviour, attachment and metamorphosis in black-footed abalone (*Haliotis iris*) larvae. *New Zealand Journal of Marine and Freshwater Research*, 48(3), 314–334.
- Baloun, A. J., & Morse, D. E. (1984). Ionic control of settlement and metamorphosis in larval *Haliotis rufescens* (Gastropoda). *Biological Bulletin*, 167, 124–138.
- Barlow, L. A. (1990). Electrophysiological and behavioral responses of larvae of the red abalone (*Haliotis rufescens*) to settlement-inducing substances. *Bulletin of Marine Science*, 46(2), 573–554.
- Bryan, P. J., & Qian, P. (1998). Induction of larval attachment and metamorphosis in the abalone *Haliotis diversicolor* (reeve). *Journal of Experimental Marine Biology and Ecology*, 223, 39–51.
- Burke, C., Harris, J. O., Hindrum, S. M., Edwards, S. J., & Maguire, G. B. (2001). Growth reduction in greenlip (*Haliotis laevis*) and blacklip (*H. rubra*) abalone resulting from chronic exposure to sublethal combination of elevated ammonia and low dissolved oxygen levels. *Environmental Requirements of Abalone*, 97, 48–57.
- DAFF. (2016). Aquaculture yearbook 2016 south Africa (Vol. 60, Issue 4). <https://doi.org/10.1007/BF03396777>
- Daume, S., Brand-Gardner, S., & Woelkerling, W. J. (1999). Preferential settlement of abalone larvae: Diatom films vs. non-geniculate coralline red algae. *Aquaculture*, 174(3–4), 243–254.
- de Waal, S. W. 2002. Factors influencing the ranching of the abalone species *Haliotis midae* along the namaqualand coast of South Africa. PhD Thesis, University of Cape Town, 1–254. <http://hdl.handle.net/11427/10424>
- Ersts, P. J. (2019). DotDotGoose (version 1.1.1) American Museum of Natural History, Centre for Biodiversity and Conservation. https://biodiversityinformatics.amnh.org/open_source/dotdotgoose
- Fox, J., & Weisberg, S. (2019). *An R companion to applied regression* (3rd ed.). Sage. <https://socialsciences.mcmaster.ca/jfox/Books/Companion/>
- Gapasin, R. S. J., & Polohan, B. B. (2004). Induction of larval settlement and metamorphosis in the donkey-ear abalone, *Haliotis asinina* Linnaeus, by chemical cues. *Hydrobiologia*, 519, 9–17.
- Godfrey, B. P. 2003. The potential of abalone stock enhancement in the Eastern Cape Province of South Africa. MSc Thesis, Rhodes University, 1–163. <http://core.ac.uk>
- Gordon, N., Shpigel, M., Harpaz, S., Lee, J. J., & Neori, A. (2004). The settlement of abalone (*Haliotis discus hannai* Ino) larvae on culture layers of different diatoms. *Journal of Shellfish Research*, 23(2), 1–36.
- Hothorn, T., Bretz, F., & Westfall, P. (2008). Simultaneous inference in general parametric models. *Biometrical Journal*, 50(3), 346–363.
- Huggett, M. J., De Nys, R., Williamson, J. E., Heasman, M., & Steinberg, P. D. (2005). Settlement of larval blacklip abalone, *Haliotis rubra*, in response to green and red macroalgae. *Marine Biology*, 147, 1155–1163.
- Kang, K. H., Kim, B. H., & Kim, J. M. (2004). Induction of larval settlement and metamorphosis of the abalone, *Haliotis discus hannai* larvae using bromomethane and potassium chloride. *Aquaculture*, 230(1–4), 249–259.
- Leighton, P. (2008). Abalone hatchery manual. *Irish Sea Fisheries Board*, 25, 1–89.
- Li, H., Wei, L., Zhang, G., Cai, Z., Cai, G., Chang, Y., & Xing, K. (2006). Enhancement of larval settlement and metamorphosis through biological and chemical cues in the abalone *Haliotis diversicolor* supertexta. *Aquaculture*, 258, 416–423.
- Liu, B., Dong, B., Tang, B., Zhang, T., & Xiang, J. (2006). Effect of stocking density on growth, settlement and survival of clam larvae, *Meretrix meretrix*. *Aquaculture*, 258, 344–349.
- Morse, A. N. C. (1991). How do planktonic larvae know where to settle? *American Scientist*, 79(2), 154–167.
- Moss, G. A. (1999). Factors affecting settlement and early post-settlement survival of the New Zealand abalone *Haliotis australis*. *New Zealand Journal of Marine and Freshwater Research*, 33(2), 271–278.
- Moss, G. A., & Tong, L. J. (1992). Techniques for enhancing larval settlement of the abalone, *Haliotis iris*, on artificial surfaces on artificial surfaces. *New Zealand Journal of Marine and Freshwater Research*, 26, 75–79.
- R Core Team. (2021). *A language and environment for statistical computing*. R Foundation for Statistical Computing.
- Raemaekers, S., Hauck, M., Bürgener, M., Mackenzie, A., Maharaj, G., Plagányi, É. E., & Britz, P. J. (2011). Review of the causes of the rise of the illegal south African abalone fishery and consequent closure of the rights-based fishery. *Ocean and Coastal Management*, 54, 433–445.
- Slattery, M. 1987. Settlement and metamorphosis of red abalone (*Haliotis rufescens*) larvae: A critical examination of mucus, diatoms and γ -aminobutyric acid (GABA) as inductive substrates. MSc thesis. San Jose State University, 1–39. <https://scholarworks.calstate.edu/downloads/5138j43q>
- Stewart, P., Soonklang, N., Stewart, M. J., Wanichanon, C., Hanna, P. J., Poomtong, T., & Sobhon, P. (2008). Larval settlement of the tropical abalone, *Haliotis asinina* Linnaeus, using natural and artificial chemical inducers. *Aquaculture Research*, 39, 1181–1189.
- Todd, C. D., Bentley, M. G., & Jonathan, N. H. (1991). Larval metamorphosis of the opisthobranch mollusc *Adalaria proxima* (Gastropoda: Nudibranchia): The effects of choline and elevated potassium ion concentration. *Journal of the Marine Biological Association of the United Kingdom*, 71(1), 53–72.

- White, H. I., Hecht, T., & Potgieter, B. (1996). The effect of four anaesthetics on *Haliotis midae* and their suitability for application in commercial abalone culture. *Aquaculture*, 140(1-2), 145-115.
- Witte, W. 2017. Abalone stock enhancement at Cape Recife, eastern cape province of South Africa. MSc Thesis, Rhodes University, 1-168. <http://hdl.handle.net/10962/45695>.
- Yool, A. J., Grau, S. M., Hadfield, M. G., Jensen, R. A., Markell, D. A., & Morse, D. E. (1986). Excess potassium induces larval metamorphosis in four marine invertebrate species. *The Biological Bulletin*, 170(2), 255-266.
- Yu, X., Yan, Y., & Li, H. (2010). The effect of chemical cues on larval settlement of the abalone, *Haliotis diversicolor* super-texta. *Journal of the World Aquaculture Society*, 41(4), 626-632.

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Appendix 4 Published research article

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Check for updates

Staining of larval abalone (*Haliotis midae*) shells using two epifluorescent dyes

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ABSTRACT

A nontoxic method that results in distinct and persistent marks in *Haliotis midae* juveniles is fundamental for field-based studies, abalone ranching, and stock enhancement programs. Developed (6 days after fertilization) *H. midae* larvae were batch-tagged with 25 and 50 mg L⁻¹ of calcein and xylene orange for 24 and 48 h to determine the effect on survival, settlement, and the persistence of the mark on the shell. Epifluorescence was detected in the shell spire of larvae tagged with either 25 mg L⁻¹ or 50 mg L⁻¹ of calcein for 24 and 48 h immediately after the staining (t_0) and was visible after 51 days when the experiment was terminated. Survival of calcein stained larvae was higher after 24 h compared to 48 h immersion. However, percentage settlement was higher in the 48 h treatments when compared to 24 h treatments for both concentrations and dyes. Therefore, bright and persistent epifluorescence marks can be induced in *H. midae* shells using calcein (25 mg L⁻¹ for 24 h) without negatively affecting the survival and settlement of larvae, post-immersion.

KEYWORDS

Calcein; xylene orange; settlement; persistent marking; batch-tag

Introduction

The South African wild abalone (*Haliotis midae*, commonly known as perlemoen) fishery has experienced a considerable decline in population abundance over the last three decades, primarily due to heavy fishing pressure from the illegal catch (Witte 2017). Two techniques have been evaluated to restore depleted stocks of this commercially valuable species. Stock enhancement using cultured juveniles has been practiced in Japan for several decades, while pilot-scale feasibility studies have been conducted in South Africa (Godfrey 2003; Witte 2017). The associated high cost of seeding juvenile abalone has led to an interest in the possibility of larval seeding (Preece et al. 1997). Studies on the larval seeding of abalone have been conducted on a small-scale, experimental level (Mills-Orcutt, Bouma, and Donovan 2020; Preece et al. 1997; Tong, Moss, and Illingworth 1987) on a variety of species. Results have shown high variation in survival for the different species seeded

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which is likely influenced by the different seeding methods, suggesting that strategies for out-planting larvae are probably site and species-specific.

When conducting field-based studies or stock enhancement programs, released individuals must be unambiguously identifiable from wild animals, and therefore, a reliable, effective tagging method is required. However, tagging methods to identify individuals uniquely can often be problematic due to extensive handling, which is also labor intensive if a large number is tagged and can also negatively affect the survival and growth of the individuals (Chick 2010). Free swimming abalone larvae are small in size (*H. midae* $207 \times 265 \mu\text{m}$) (Genade, Hirst, and Smit 1988), which makes it challenging to tag them individually, and larvae are also sensitive to handling and manipulation (Chick 2010). The batch-tagging method is an effective method for tagging many individuals simultaneously. The marking tag can either be added to water in which the animals are then marked by immersion, or it can be incorporated into the feed (Chick 2010). The immersion marking method is the most suitable for small life stages (Purcell and Blockmans 2009), such as abalone larvae, since they may not begin feeding until settlement.

Fluorochromes are calcium-binding substances that are incorporated at the site of active mineralization of bone, known as calcification front at the site of new bone formation (Day, Williams, and Hawkes 1995; Kaehler and McQuaid 1999; Moran 2000; Rahn and Perren 1971; Stuart and Smith 1992). After binding, the dye provides a bright epifluorescence reference which remains embedded in the growing shell at the top of the spire (Chick 2010). Fluorochrome dyes have been used for batch-tagging small life stages such as class Gastropoda abalone larvae (Chick 2010; Day, Williams, and Hawkes 1995), snails (Moran 2000) and class Bivalvia (van der Geest et al. 2011). Studies have shown that fluorochrome dye does not appear to have a detrimental effect on aquatic larvae when administered at an appropriate concentration (Day, Williams, and Hawkes 1995; Moran 2000; Moran and Marko 2005; Uggeri et al. 2004) and extended immersion periods at the appropriate concentration can result in higher levels of epifluorescence brightness (Purcell and Blockmans 2009). However, fluorochrome dyes can be toxic in marine animals if used at excessively high concentrations for a prolonged period (Purcell and Blockmans 2009).

Fluorochromes commonly used for marking the shell of marine species include calcein, xylenol orange, alizarin red S, oxytetracycline, and tetracycline. Fluorochrome calcein is a nonfluorescent, cell-permeant compound converted by intracellular esterases into calcein, the anionic fluorescent form that fluoresces bright green when combined with calcium (Uggeri et al. 2004). Calcein has been used successfully to mark the shell of different species in numerous growth studies, i.e., abalone larval, *Haliotis rubra* (Chick 2010; Day, Williams, and Hawkes 1995), juvenile scallop, *Amusium balloti* (Lucas et al. 2008); green-lipped mussel larvae,

Perna canaliculus (Fitzpatrick, Jeffs, and Dunphy 2013); and the bivalve, *Loripes lacteus* (van der Geest et al. 2011). The calcein mark retained within the shell during settlement and metamorphosis remain embedded in the growing shell at the top of the spire (Collin and Voltzow 1998). Xylenol orange is fixed in newly formed calcified tissues where it remains throughout the growth of the animal (Rahn and Perren 1971) and has been successfully used to mark abalone larvae, *H. rubra* (Day, Williams, and Hawkes 1995).

The release of hatchery-produced larvae has been identified as an alternative option to building depleted wild abalone stock; millions of larvae can be produced in a hatchery on demand and cost-effectively. However, research still needs to be done to test the viability of using abalone larvae in terms of potential economic benefit, including tracking the survival and growth of released larvae. One of the critical factors required to determine the feasibility of this stock enhancement technique is the unambiguous identification of the released abalone from the wild stock. Marked recapture allows quantification of fundamental aspects of biology such as mortality, growth, population size, age at maturity, and possible track movement, which is essential information for stock enhancement programs (Lucas et al. 2008). Identifying hatchery-produced larvae will also enable proof of resource ownership for abalone ranching programs, which is also critical for the further development of abalone ranching. This study aimed to compare and identify an ideal dosage of two fluorochrome dyes (calcein and xylenol orange) in staining the shell of abalone (*H. midae*) larvae and assessing their effect on initial survival and settlement as well as brightness and longevity of resultant mark. The objectives of this study were to determine (1) whether the concentration of the chemical dyes have any effect on larval survival and settlement; and (2) if the combination of immersion time and epifluorescence dye concentrations can induce a persistent marker in the larval shell which is detectable using fluorescent light. The results from this study could be used in abalone larvae reseeding or stock-enhancement programs to help identify the hatchery-produced juveniles upon recapture.

Materials and methods

The study was conducted at Wild Coast Abalone Farm (Pty) Ltd (32°45'05"S 28°16'27" E), South Africa. Abalone larvae were raised at the WCA farm hatchery in a flow-through rearing system, with each aquarium receiving independent aeration and filtered (1 µm) UV-treated seawater. Larvae were developed until they reached a stage where they could settle, 6 days after fertilization. Two fluorochrome dyes were calcein-AM (Sigma-Aldrich, Johannesburg, South Africa) and xylenol orange tetrasodium salt (Reflecta Laboratory Supplies, Johannesburg, South Africa).

Experimental setup

The experiment was conducted in 20 L tanks filled with 1 μm filtered seawater mixed with sodium bicarbonate (0.1 g. L^{-1}) to buffer the pH in the seawater. Calcein and xylenol orange were used as fluorescent markers with each at a concentration of either 25 mg. L^{-1} or 50 mg. L^{-1} . The chemical powder was dissolved in the water before adding the abalone larvae. Larvae were held in each tank for 24 h and 48 h, with aeration but without water flow. Two controls containing no fluorochrome dye for the 24 h and 48 h periods were included. All treatments had three replicates.

Approximately 40,000 six-day-old larvae that were competent to settle were collected from the larval rearing tanks onto an $80 \mu\text{m}$ screen and transferred into the tanks (2 larvae mL^{-1}) containing calcein or xylenol orange. Larvae were immersed in the marking baths for 24 h and 48 h, with both treatments starting at the same time (t_0), and then held for their respective immersion times (24 h and 48 h). The tanks were in a temperature-controlled room maintained at $18 \pm 0.5^\circ\text{C}$.

Evaluating the effect of calcein and xylenol orange immersion period on abalone larval survival

Immediately after the respective immersion periods, patches of dead larvae, which tend to accumulate in dark clumps at the bottom of the rearing tank (referred to in abalone hatcheries as “spotting”), were siphoned from each tank. The remaining larvae were gently poured through an $80 \mu\text{m}$ screen and placed into a tank with flow-through water to remove excess dye. Once clean, the larvae were gently poured into 10 L tanks containing filtered ($1 \mu\text{m}$) seawater. Each tank received water (6 L per hour) and gentle aeration. In each tank, two plastic sheets ($15 \times 15 \text{ cm} = 900 \text{ cm}^2$ total surface area per tank) pre-coated with natural diatom film were suspended as inducers for settlement. The sheets had been conditioned by placing them in outside abalone weaning tanks supplied with incoming seawater and allowed to develop a film of natural diatoms.

Post-exposure larval survival was determined by taking 10 mL water samples from each tank in triplicate. The number of swimming larvae was counted under a dissecting microscope (Best Scope, NSZ-606) as a function of the initial stocking density (2 larvae per mL).

Determining the effect of calcein and xylenol orange immersion period on abalone larvae initial settlement

To determine the effect of calcein and xylenol orange on the settlement of larvae at different concentrations and immersion times, after 24 and 48 h post-transfer to the clean 10 L tank, each sheet was removed and photographed (Canon camera, EOS 2000D) at high resolution (≈ 24.1 megapixels) so that the number of settled larvae could be counted on both sides of each sheet. The experiment ended after 48 h because the settlement sheet was small and

insufficient to provide food for an extended period. The data presented in was recorded after 48 h and 24 hours post 24- and 48-h immersion periods, respectively, to ensure that larvae were in the same developmental stage.

Determining the persistence of epifluorescent dose in the shell of juvenile *H. midae*

The experiment was conducted following the same method described in the first experiment to determine the persistence of calcein and xylene orange stain in the shell of juvenile *H. midae*. After the respective immersion period, 3500 and 4500 larvae were placed into small (26 cm²) and large (31.8 cm²) abalone settlement bags, respectively. Bags were randomly placed in the hatchery larval culture room with two replicates for each treatment. The inside of the bags were coated by filling the plastic bag with seawater, and allowing a natural diatom film to attach and grow in the bags for 2 weeks before the experiment. The stained larvae were then introduced into the bags where they settled and started feeding on the diatom layer. During the first 5 days of settlement, the water flow was switched off to avoid flushing the swimming larvae out of the bags. Each bag received aeration while the temperature was maintained at $18 \pm 0.2^\circ\text{C}$. Larvae were raised in the settlement bags until food became limiting which was at 51 days after transfer into the bag. The persistence and level of epifluorescence were determined by taking 15 individuals from each bag at t_0 (start of the experiment), and subsequent samples were collected after 24-days, 38-days, 47-days, and 51-days post-transfer to the bags. Samples were stored at -20°C , and after thawing, the shell of each juvenile was observed under a fluorescence microscope (Olympus BX-61) using the FITC set (green fluorescent), DAPI set (blue fluorescent) and Red set (Red fluorescent) to visualize the larval shell. Images of the abalone shell were captured using a Leica DFC 490 camera.

Images were then analyzed for brightness, where the intensity of fluorochrome (brightness of the band) was quantified using “ImageJ” image processing software’s freehand selection tool and histogram analysis function (developed by Wayne Rasband at the National Institutes of Health (NIH)). This function quantifies the brightness of each pixel within the selected area. The number of pixels for each value is plotted on a histogram, and the overall mean brightness of the selected area is established. The mean brightness was recorded to measure the total chemical fluorescence for each abalone’s shell band (Fitzpatrick, Jeffs, and Dunphy 2010).

Statistical analysis

All data were analyzed to check if they met parametric test assumptions using Shapiro–Wilk test for normality and Levene’s test for homogeneity. To test the effect of different concentrations of the fluorochrome dyes on larval survival and initial settlement, Levene’s test revealed non-homogeneity of variances and the data were log transformed. Then the data were analyzed using Welch’s

analysis of variance (ANOVA), which is robust to non-equal variance among treatments. The mean epifluorescence brightness between fluorochrome (exposure period and different concentrations) met the parametric test assumption and was analyzed using a one-way ANOVA. For all data, when significant differences ($p < .05$) were found, Tukey's multiple comparisons test was used to determine specific differences among treatments. An independent sample t -test was also used to compare the overall mean between exposure period treatments. The IBM SPSS version 26 statistical program was used for all data analyses with all data presented as mean $\pm$ SE.

Results

Determining the effect of calcein and xylenol orange immersion period on abalone larval survival and initial settlement

Survival of *H. midae* larvae was significantly affected by both immersion periods and different concentrations of fluorochromes. Abalone larvae immersed for 24 hours had an overall higher percentage survival than those exposed to 48 hours immersion ($t(28) = 7.96, p < .001$). After 24 h immersion, the highest percentage survival of $86.78 \pm 4.83\%$ and $80.65 \pm 10.54\%$ was recorded in the xylenol orange 50 mg L^{-1} and 25 mg L^{-1} treatments, respectively. The overall survival of abalone larvae declined after 48 h immersion for both fluorescent marker treatments with survival below 35%. The highest percentage survival of $32.54 \pm 1.17\%$ and $23.73 \pm 1.50\%$ were recorded for xylenol orange 25 mg L^{-1} and calcein 50 mg L^{-1} , respectively, while xylenol orange 50 mg L^{-1} had the lowest survival ($2.35 \pm 0.98\%$) compared to the other treatments, including the control (Figure 1).

Determining the effect of calcein and xylenol orange immersion period on abalone larvae initial settlement

The initial settlement of abalone larvae was significantly affected by the immersion period and different concentrations of fluorochrome dyes. The immersion period had a significant effect on the percentage settlement of *H. midae* larvae, with the overall higher percentage of settled larvae recorded (averaged across fluorescent marker type) after 48 h immersion ($5.89 \pm 0.58\%$) compared to 24 h immersion ($2.25 \pm 0.55\%$) ($t_{(78)} = -4.45, p < .001$). After 24 h immersion, there was no difference in the larval percentage settlement. While after 48 h immersion period, the percentage settlement of larvae was different between treatments ($F_{(4)} = 46.02, p < .001$). After 48 h immersion, the xylenol orange 50 mg L^{-1} treatment had the highest percentage settlement, followed by calcein 25 and 50 mg L^{-1} , while the control treatment had the lowest settlement (Figure 2).

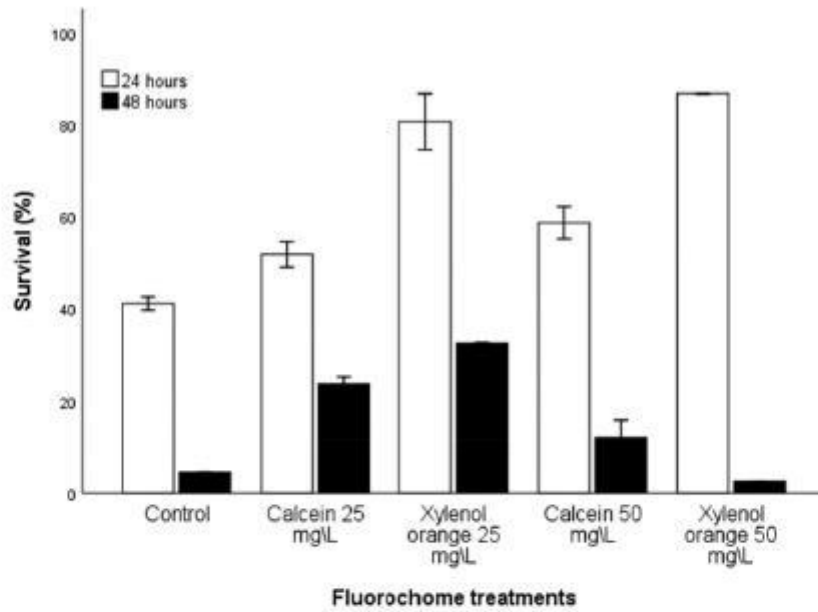


Figure 1. Percentage survival ($\pm$ SE) of *haliotis midae* larvae immediately after 24 and 48 h immersion periods in calcein and xylenol orange at concentrations of 25 mg. L⁻¹ and 50 mg. L⁻¹.

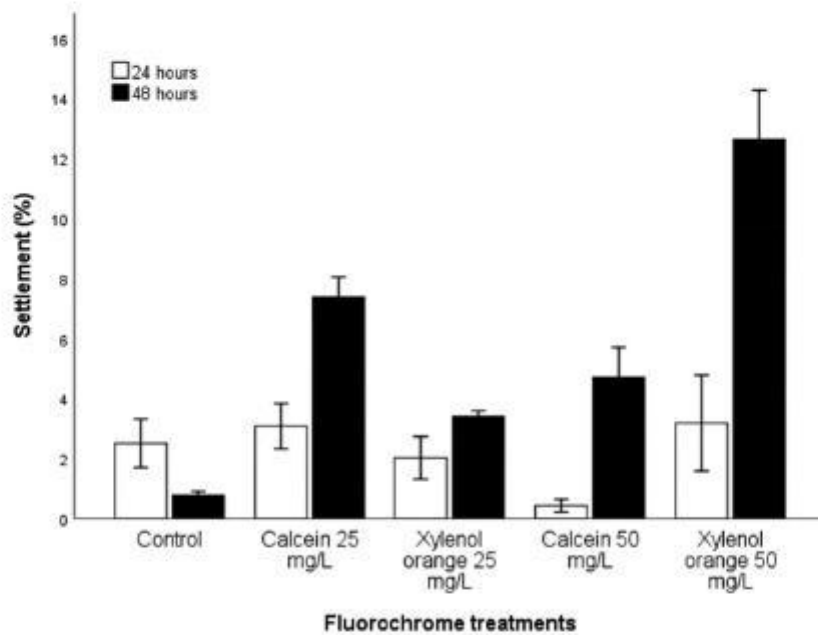


Figure 2. Percentage settlement ($\pm$ SE) of *haliotis midae* larvae after immersed in calcein and xylenol orange at concentrations of 25 mg. L⁻¹ and 50 mg. L⁻¹ for 24 and 48 h period.

Determining the persistence of epifluorescence in the shell of juvenile *H. midae*

There was 100% marking success of larvae immersed in the calcein treatment at both concentrations and immersion periods (Figure 3). Epifluorescence bands were evident in all juvenile abalone shells after 51 days. The control treatments also had no natural epifluorescence and there was no successful marking of larval shells in either of the xylenol orange treatments. Higher and more consistent levels of mean brightness were observed in the 50 mg L⁻¹ calcein batch-tagged juvenile shells, irrespective of the immersion period ($F(3) = 137$, $p < .001$). The difference in the mean brightness among calcein concentrations varied with immersion time, and 48 h had highest mean brightness level of both concentrations ($t(397) = 2.182$, $p = .030$). Larvae immersed for 48 h at 50 mg L⁻¹ calcein had highest mean brightness (199.59

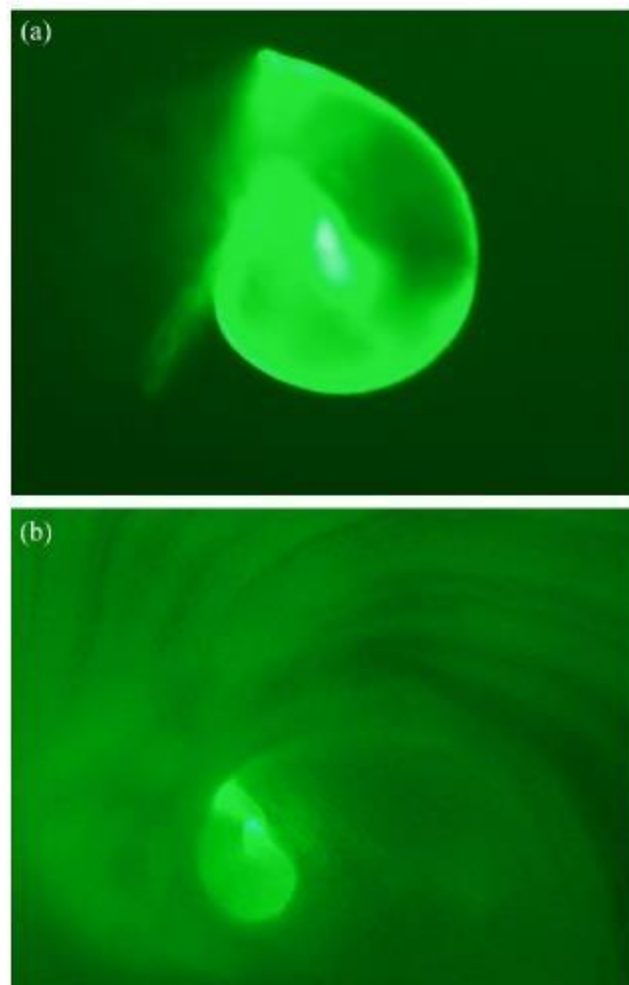


Figure 3. (a,b) photomicrographs of the spire of a calcein-marked 7day-old larvae *haliotis midae* viewed under a fluorescence microscope (a) and showing the spire on the shell of juvenile abalone 51 day-old post settlement (b).

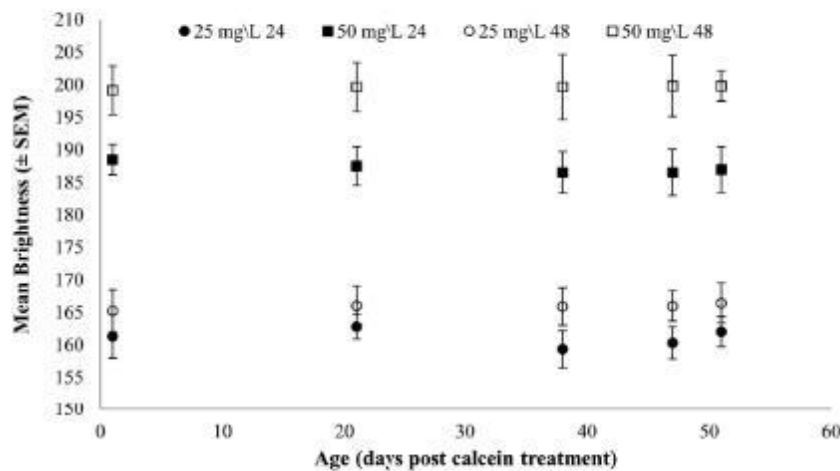


Figure 4. Mean brightness ($\pm$ SEM) of fluorochrome on the shell of juvenile *haliotis midae* immersed for 24 and 48 h at two concentrations (25 and 50 mg L⁻¹) of calcein.

± 1.57 SEM) compared to those immersed for 24 h at 25 mg L⁻¹ (161.16 $\pm$ 1.63 SEM). There was no difference in the level of mean brightness associated with time (decrease of the mean brightness level) of *H. midae* juveniles sampled before and post-settlement (Figure 4).

Discussion

The choice of fluorescent dye for batch-tagging abalone larvae should not be based solely on tag brightness; post-staining survival is also essential. Larval survival is crucial for both abalone hatcheries and stock enhancement programme. Longer immersion (48 h) of *H. midae* larvae on calcein or xylenol orange affected the survival, ranging between 10% and 20% compared to 40% and 95% after 24 h immersion, respectively. This survival percentage was different from that found by Chick (2010), who observed that staining *Haliotis rubra* larvae with calcein does not appear to affect larval survival. These differences could be attributed to (1) different methods used in these studies to determine survival, Chick (2010) is based on assumptions while survival was measured in this study. (2) Species-specific abalone settlement variability may have resulted in some larvae settling on the side surface of the tank after 48 h immersion; hence, fewer larvae were swimming in the water column.

Fluorescent dyes can be toxic to marine organisms (Day, Williams, and Hawkes 1995) when administered at high concentrations or for extended periods (Purcell and Blockmans 2009). Fluorescent dyes positively affected the survival of *H. midae* larvae, and in both immersion periods, survival was higher in all treatments than in control. Similar results were reported by Moran and Marko (2005), who tested the survival of bivalve larvae *Mytilus*

trossulus marked with calcein which was significantly higher in 7-day post-marked larvae ($38 \pm 3\%$ (SD)) compared to unmarked larvae ($21 \pm 4\%$ (SD)). The underlying mechanism for this positive effect on survival is unknown. Moran and Marko (2005) stated that one of the significant causes of mortality in larval culture is microbiological contamination, predominantly bacteria and viruses. While microorganism concentration was not evaluated in this study, pausing UV-treated water flow during the staining period might increase the chance for microorganism growth. The positive effect of calcein and xylenol orange on the survival of *H. midae* larvae suggests that both fluorochrome dyes possibly had an antibiotic or antiviral effect.

Abalone larvae must survive the planktonic stage and seek a suitable substrate where they change from swimming larvae into benthic juveniles (Leighton 2008). All abalone larvae settled post-immersion across all treatments. However, more extended immersion periods influenced the settlement of *H. midae* larvae, resulting in a higher percentage settlement. This may have resulted from the stress caused to abalone larvae during the longer (48 h) immersion periods. Larvae (*Haliotis laevis*) are sensitive to poor water quality which stresses the abalone and slows growth, in the worst case, water quality can deteriorate to the extent of causing mortalities (Hindrum et al. 2001). While Yu, Yan, and Li (2010) reported that abalone larvae fall out of suspension and consequently attach to the substratum under the stressed condition. After 48 h immersion, *H. midae* were possibly stressed, forcing more larvae to settle. It is possible that abalone larvae were more developed after 48 h immersion which might have resulted in higher percentage settlement (Moss and Tong 1992).

Different fluorescent dye concentrations also play a vital role in settlement of *H. midae* larvae. While there was no difference between the treatments and the control after 24 h immersion, xylenol orange (50 mg. L^{-1}) and calcein (50 mg. L^{-1}) had higher settlement after 48 h immersion. It has been shown in previous small-scale experiments that abalone larvae can continue to settle for up to 94 h post-initial settlement (Mzozo, Hugo, and Vine 2023). Unfortunately, the percentage settlement of *H. midae* post-settlement was not measured beyond 48 h. It is, therefore, unclear what might have happened with unsettled larvae from the control treatment. Therefore, conclusions on the effect of fluorescent dye on the long-term settlement of *H. midae* larvae should be made with caution.

During the batch-tagging of abalone larvae, the epifluorescence mark is embedded in the spiral of the growing shell of abalone (Chick 2010). The incorporation of epifluorescent markers in the shell increases with an increase in dye concentration of fluorescent and immersion period (Pirker and Schiel 1993). This study showed that the use of calcein enabled unambiguous identification of tagged *H. midae* larvae from untagged larvae (control treatment) post immersion, and the mark is a visible bright green, fluorescent FITC band

on the shell when observed under the fluorescence microscope. Chick (2010) reported that *H. rubra* juveniles batch-tagged with calcein demonstrated a visible mark was in the shell spire regardless of the concentration or immersion period. The results of this study clearly indicate staining *H. midae* larvae with 50 mg L⁻¹ calcein results in a brighter epifluorescent mark on the shell than using 25 mg L⁻¹, regardless of the immersion period. Similar results were obtained by Day, Williams, and Hawkes (1995), who reported that fluorescent marks in the shell of abalone juveniles, *H. rubra* stained with calcein, were brightest at a concentration of 60 mg L⁻¹ compared to 10 mg L⁻¹ irrespective of the immersion time. Furthermore, van der Geest et al. (2011) reported that the percentage of successfully marked bivalve, *Loripes lacteus* increased with higher calcein concentrations (100 – 800 mg L⁻¹).

The most appropriate protocol for quality marking may vary between species (Eads and Layzer 2002). The xylenol orange treatments did not produce a mark on the shell of *H. midae* larvae when reviewed under the FITC filter sets of the fluorescence microscope. These results contradict the claims of Day, Williams, and Hawkes (1995), who reported *H. rubra* larvae marked with xylenol orange at concentrations between 20 and 120 mg L⁻¹ produced a visible mark in the shell after 48 h immersion. The method used by Day, Williams, and Hawkes (1995) was different from this study in that the *H. rubra* shells were put through a series of water, acetone, and resin baths to facilitate resin penetration. The shell was then embedded in non-fluorescing resin before being viewed under a fluorescence microscope. This may have helped increase the contrast or brightness of the mark, making it easier to be detected by fluorescence microscope. Other research has used xylenol orange to mark dermal spicules of sea cucumbers (Purcell and Blockmans 2009) and the bone of rabbit (Rahn and Perren 1971) supporting the use of methods that do not implement embedding samples in resin. Day, Williams, and Hawkes (1995) suggested that 60 mg L⁻¹ was the best concentration for *H. rubra*; so it is possible that the xylenol orange concentrations used in this study were too low to result in a visible mark in the shell of *H. midae* juveniles. Eads and Layzer (2002) reported that the marking success of batch tags varies between species. The authors showed that calcein (250 mg L⁻¹) resulted in a distinct marking with rings around the whole shell for bivalves *Actinonaias pectorosa* while the marking was indifferent to that of *Lampsilis cardium*.

The persistence of an epifluorescence mark in the abalone shell is significant for the long-term use of the marking methodology (Eads and Layzer 2002). Consistency in the intensity epifluorescence is important when batch tagging for stock enhancement, as all marked individuals must be quickly identified among wild stock (Fitzpatrick, Jeffs, and Dunphy 2013). Calcein-produced epifluorescent marks were clearly visible on tagged *H. midae* juveniles raised under culture conditions after 51 days. Also, there was no difference in the mean brightness of epifluorescence between larvae (before settlement) and juveniles

after 51 days post-immersion, suggesting there was no epifluorescence loss over the period. Similar results were obtained by Chick (2010), who reported that up to 95% of *H. rubra* larval shells were easily distinguishable and that there was no difference in the level of epifluorescence after 260 days. Although this experiment was terminated after 51 days due to food limitations in the settlement bags, this study suggests that calcein at concentrations of 25 and 50 mg L⁻¹ are suitable for the successful long-term tagging of *H. midae* juveniles.

In conclusion, it is possible to produce bright and persistent epifluorescence marks using calcein in *H. midae* shells that do not negatively affect the larvae's survival during immersion or at settlement. A 24-h calcein immersion period results in higher larvae survival and is recommended as it subsequently increases the chances of settlement. Although 50 mg L⁻¹ produced high mean brightness, at 25 mg L⁻¹ it was still clearly visible and persistent after 51 days, making it easy to identify marked individuals. Calcein is expensive, and therefore, if budget is limited, 25 mg L⁻¹ is recommended over higher concentrations (50 mg L⁻¹). These results are of value to studies investigating larval dispersal dynamics and where larvae are released and recaptured, such as stock enhancement or ranching programs. Further research should further refine the optimal calcein concentration to create a persistent mark as well as determine the long-term effect of calcein staining on the survival and settlement of early-stage juvenile *H. midae* in the natural environment.

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References

- Chick, R. 2010. Batch-tagging blacklip abalone (*haliotis rubra*) for identification of hatchery-reared individuals on natural coastal reefs in new South Wales, Australia. *Journal of Shellfish Research* 29 (1):209–15. doi:10.2983/035.029.0117.
- Collin, R., and J. Voltzow. 1998. Initiation, calcification, and form of larval “archaeogastropod” shells. *Journal of Morphology* 235 (1):77–89. doi:10.1002/(SICI)1097-4687(199801)235:1<77::AID-JMOR6>3.0.CO;2-L.
- Day, R., M. Williams, and G. Hawkes. 1995. A comparison of fluorochromes for marking abalone shells. *Marine and Freshwater Research* 46 (3):599–605. doi:10.1071/mf9950599.
- Eads, C., and J. B. Layzer. 2002. How to pick your mussels out of a crowd: Using fluorescence to mark juvenile freshwater mussels. *The University of Chicago Press Journals* 21 (3):476–86. doi:10.2307/1468484.
- Fitzpatrick, M. P., A. G. Jeffs, and B. J. Dunphy. 2010. Identification of the optimal fluorochrome for marking larvae of the pulmonate limpet *siphonaria australis*. *Journal of Shellfish Research* 29 (4):941–44. doi:10.2983/035.029.0427.
- Fitzpatrick, M. P., A. G. Jeffs, and B. J. Dunphy. 2013. Efficacy of calcein as a chemical marker of green-lipped mussel (*Perna canaliculus*) larvae and its potential use for tracking larval dispersal. *Aquaculture Research* 44 (3):345–53. doi:10.1111/j.1365-2109.2011.03034.x.
- Genade, A. B., A. L. Hirst, and C. J. Smit. 1988. Observations on the spawning, development and rearing of the South African abalone *haliotis midae* linn. *South African Journal of Marine Science* 6 (1):3–12. doi:10.2989/025776188784480465.
- Godfrey, B. P. 2003. The potential of abalone stock enhancement in the Eastern Cape province of South Africa. MSc Thesis., *Rhodes University*. 1–170. <http://core.ac.uk>
- Hindrum, S. M., C. Burke, and S. J. Edwards. 2001. Growth reduction in greenlip (*haliotis laevis*) and blacklip (*H. rubra*) abalone resulting from chronic exposure to sublethal combination of elevated ammonia and low dissolved oxygen levels. *Environmental Requirements of Abalone* 48–57.
- Kaehler, S., and C. D. McQuaid. 1999. Use of the fluorochrome calcein as an in situ growth marker in the brown mussel *Perna perna*. *Marine Biology* 133 (3):455–60. doi:10.1007/s002270050485.
- Leighton, P. 2008. Abalone hatchery manual. *Irish Sea Fisheries Board* 1–89.
- Lucas, T., P. J. Palmer, S. Wang, R. Scoones, and E. O'Brien. 2008. Marking the shell of the saucer scallop *amusium balloti* for sea ranching using oxytetracycline, calcein and alizarin red S. *Journal of Shellfish Research* 27 (5):1183–88. doi:10.2983/0730-8000-27.5.1183.
- Mills-Orcutt, K. A., J. V. Bouma, and D. A. Donovan. 2020. Outplanting larval pinto abalone *Haliotis kamtschatkana* kamtschatkana (Jonas) as a recovery tool in the Salish sea. *Journal of Shellfish Research* 39 (2):381–88. doi:10.2983/035.039.0220.
- Moran, A. L. 2000. Calcein as a marker in experimental studies newly-hatched gastropods. *Marine Biology* 137 (5–6):893–98. doi:10.1007/s002270000390.
- Moran, A. M. Y. L., and P. B. Marko. 2005. A simple technique for physical marking of larvae of marine bivalves. *Journal of Shellfish Research* 24:567–71. doi:10.2983/0730-8000(2005)24[567:ASTFPM]2.0.CO;2.
- Moss, G. A., and L. J. Tong. 1992. Effect of stage of larval development on the settlement of the abalone, *Haliotis iris*. *New Zealand Journal of Marine and Freshwater Research* 26 (1):69–73. doi:10.1080/00288330.1992.9516501.
- Mzozo, Z. B., S. Hugo, and N. G. Vine. 2023. The use of chemical and biological settlement cues in enhancing the larval settlement of abalone (*haliotis midae*): Implications for hatcheries and ocean ranching. *Journal of the World Aquaculture Society* 54 (6):1702–17. doi:10.1111/jwas.13001.

- Pirker, J. G., and D. R. Schiel. 1993. Tetracycline as a fluorescent shell-marker in the abalone *halotis iris*. *Marine Biology* 116 (1):81–86. doi:10.1007/BF00350734.
- Preece, P. A., S. A. Shepherd, S. M. Clarke, and J. K. Keesing. 1997. Abalone stock enhancement by larval seeding: Effect of larval density on settlement and survival. *Molluscan Research* 18 (2):265–73. doi:10.1080/13235818.1997.10673700.
- Purcell, S. W., and B. F. Blockmans. 2009. Effective fluorochrome marking of juvenile sea cucumbers for sea ranching and restocking. *Aquaculture* 296 (3–4):263–70. doi:10.1016/j.aquaculture.2009.08.027.
- Rahn, B. A., and S. M. Perren. 1971. Xylenol orange, a fluorochrome useful in polychrome sequential labeling of calfiying tissue. *Stain Technology* 4 (3):125–29. doi:10.3109/10520297109067836.
- Stuart, A. A. J., and D. A. Smith. 1992. Use of the fluorochromes xylenol orange, calcein green, and tetracycline to document bone deposition and remodeling in healing fractures in chickens. *American Association of Avian Pathologists* 36 (2):447–49. doi:10.2307/1591527.
- Tong, L. J., G. A. Moss, and J. Illingworth. 1987. Enhancement of a natural population of the abalone, *halotis iris*, using cultured larvae. *Aquaculture* 62 (1):67–72. doi:10.1016/0044-8486(87)90185-2.
- Uggeri, J., R. Gatti, S. Belletti, R. Scandroglio, R. Corradini, B. M. Rotoli, and G. Orlandini. 2004. Calcein-AM is a detector of intracellular oxidative activity. *Histochemistry and Cell Biology* 122 (5):499–505. doi:10.1007/s00418-004-0712-y.
- van der Geest, M., J. A. van Gils, J. van der Meer, H. Oiff, and T. Piersma. 2011. Suitability of calcein as an in situ growth marker in burrowing bivalves. *Journal of Experimental Marine Biology and Ecology* 399 (1):1–7. doi:10.1016/j.jembe.2011.01.003.
- Witte, W. 2017. Abalone stock enhancement at Cape Recife, Eastern Cape province of South Africa. MSc Thesis., *Rhodes University*. 1–168. <http://hdl.handle.net/10962/45695>
- Yu, X., Y. Yan, and H. Li. 2010. The effects of chemical cues on larval settlement of the abalone, *halotis diversicolor supertexta*. *Journal of the World Aquaculture Society* 41 (4):625–632. doi:10.1111/j.1749-7345.2010.00403.x.