Factors influencing the reproductive development and early life history of blacklip (*Haliotis rubra*) and greenlip (*H. laevigata*) abalone

by

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Abstract

A study was initiated to determine the effect of selected factors on the reproductive development and early life history of blacklip (*Haliotis rubra*) and greenlip (*H. laevigata*) abalone relevant to their wild fisheries or aquaculture. In both species, the rate of gonadal and larval development was proportional to water temperature, but the relationship was not simply multiplicative, rather there was a critical minimum water temperature below which development was arrested, known as the Biological Zero Point (BZP). The BZP for gonadal development was 7.8ºC for *H. rubra* and 6.9ºC for *H. laevigata*. Corresponding BZP values for larval development were 7.8ºC and 7.2ºC, respectively. Observations of larval development relative to temperature enabled a description of the Effective Accumulative Temperature (EAT; the cumulative difference between the culture temperature and the BZP, calculated hourly) for prominent developmental stages. The difference between the EAT for metamorphic competence and that for hatchout (i.e. the interval during which the larvae remain in the water column) was 1120 and 1160 EATºC-h for blacklip and greenlip abalone, respectively. These values, in combination with water temperature data, enable the prediction of the dispersal window for each species *in situ*. Spawning performance of blacklip and greenlip abalone was also affected by temperature, with both sexes of each species producing significantly more gametes when conditioned at 16ºC than 18ºC. Sperm production of *H. rubra* was an order of magnitude greater than that of equivalent sized *H. laevigata*. There was no apparent difference in the lipid or fatty acid composition of the ovary or testis between pre- and post-spawning animals of either species, presumably because of partial spawning and/or incomplete resorption of the gonad. Likewise, a 4ºC difference in conditioning temperature (i.e. 14ºC vs 18ºC) was insufficient to elicit changes in tissue biochemistry. There was a significant interaction between sperm density and contact time on the fertilisation success of eggs from both blacklip and greenlip abalone. Prolonged exposure (> 1200 s for *H. rubra* and > 480 s for *H. laevigata*) to concentrated sperm (i.e. 10^7 ml^{-1}) resulted in egg destruction. Analysis of CoVariance of F_{50} values (i.e. the sperm concentration required for 50%

fertilisation, derived from the linear regression of logit (proportion of eggs fertilised) versus sperm density) between species across a range of contact times demonstrated that contact time had a significant effect ($p < 0.001$) whereas species did not ($p = 0.22$). The lack of a species effect suggests that the fertilisation potential of blacklip and greenlip abalone eggs are similar, at least across the range of sperm densities and contact times used.

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Table of contents

List of tables

List of figures

Chapter 1 General Introduction

1.1 General Background

Abalone are commercially important marine gastropods (Archeogastropoda: Haliotidae) of which there are 55 extant and 40 extinct species (Geiger, 1998; Geiger and Groves, 1999) classified in a single genus *Haliotis*. They inhabit rocky reefs and boulder fields from the intertidal to a depth of 100 m (Sloan and Breen, 1988) and are native to most waters except those around South America and the Atlantic coast of North America (Hahn, 1989a). The Californian red abalone (*H. rufescens*) and the Japanese "Ezo" abalone (*H. discus hannai*) have also been introduced to Chilean waters (Flores-Aguilar, 2003).

Abalone are dioecious broadcast spawners and their life history can be broadly categorized into five stages: embryo, larvae, postlarvae, juvenile and adult. The embryonic stage typically lasts a number of hours and concludes with the hatching of the ciliated trochophore. The larvae are pelagic and lecithotrophic (i.e. non-feeding) and remain in the water column for 2-15 days depending on species and water temperature (Leighton, 1972; Sawatpeera et al., 2001). In the presence of certain chemical cues (e.g. Gamma aminobutyric acid, Morse et al., 1979) the larvae return to the benthos, metamorphose and begin to feed on benthic microflora (predominantly diatoms). From this point they are known as postlarvae. This stage lasts for about two months, during which the animal grows to 1.5-2.5 mm (Leighton, 2000). The end of the postlarval stage is signified by the appearance of the first respiratory pore. As juveniles mature, there is a gradual transition in diet from micro- to macroalgae. The size and age at which the gonad develops varies both within and between species and is dependant on factors such as temperature and food availability. Tropical species grow faster and mature earlier, but do not grow as large as temperate species. Growth of abalone during the postlarval and juvenile stages is exponential, but slows once they reach sexual maturity. The largest species, *H. rufescens* (red abalone) can reach 31 cm and almost 5 kg (Leighton, 2000).

1.2 Abalone fisheries

Abalone have been fished since early history, with the first reference to abalone divers in Japan dating from 30 A.D (Hahn, 1989b). However, it is not known whether the practice started in Japan or China. The popularity of abalone in southeast Asia spread with the gradual movement of the Chinese to other regions (e.g. Taiwan and Korea, Cuthbertson, 1978) but continuous unsustainable levels of harvesting in most of these countries has depleted or destroyed their fisheries. Abalone are particularly susceptible to overfishing owing to their ease of capture, slow growth and unpredictable recruitment (Tegner and Bulter, 1989). Other factors which impact abalone populations include El Niño events, disease and pollution. Most countries with viable abalone fisheries are attempting to reduce human impacts on abalone stocks by minimizing industrial discharge and placing restrictions (e.g. quotas, gear controls and seasonal closures) on recreational and/or commercial fisheries.

Today, the major abalone fishing nations are Australia, Japan, Mexico, New Zealand, South Africa and the United States. Recent (2002) annual catch statistics (from commercial and/or recreational fisheries) for these countries are 6062 t, 2682 t, 1616 t, 1553 t, 1281 t and 243 t, respectively (Gordon and Cook, 2004). The world market for abalone relies on about 15 species, with those sold in greatest quantities being *H. diversicolor* and *H. discus hannai*. The largest consumer nations are Japan, Hong Kong and China (through Hong Kong).

California has the oldest fishery outside of Asia, with records dating back to the mid 1800's (Cox, 1962). Intertidal and shallow water species (e.g. *H. rufescens*, *H. fulgens* and *H. cracherodii*) were collected and the meat dried for Asian markets (Leighton, 2000). By the mid 1900's, improvements in dive technology led to fishing in deeper waters, with the annual catch exceeding 2000 t until the early 1970's. However, the harvest gradually declined so that by 1996 it was less than 10% of earlier levels (Leighton, 2000). Both commercial and recreational fisheries for *H. cracherodii*, *H. sorenseni*, *H. fulgens* and *H. corrugata* were closed in 1996, and in May 1997 both sectors were excluded from fishing *H. rufescens* south of San Francisco (Leighton, 2000).

The Mexican abalone fishery relies on *H. fulgens*, *H. corrugata* and *H. cracherodii*. In the early 1920's (when records began) catches were moderate (1721 t in 1923) but by 1950 they had become unsustainably high (6000 t, Guzman del Proo, 1992). There was an immediate crash in 1951 to < 1500 t, followed by a fluctuation in catch of between 1500 t and 3500 t that continued until 1971. Seasonal closures, quotas and alterations to size limits were progressively implemented but the catch continued to decline. By 1983 it fell to < 500 t (Guzman del Proo, 1992). The catch increased again (to 2500 t) in the late 1980's and early 1990's but has since returned to around 500 t (M. Del-Rio Portillo, pers. comm.).

The modern commercial fishery for paua, *Haliotis iris*, in New Zealand dates from the mid 1940's (Schiel, 1992). Initially, only the shell was marketed (owing to its vivid colouration) but by the late 1950's both shell and meat were sold. Over-exploitation in subsequent years led to a 4-month nation-wide closure of the fishery in 1972 and a restriction on meat export in 1973 (Cooper, 1976). The ban was lifted in late 1990 (Schiel, 1992). The fishery is now managed by an individually transferable quota (ITQ) system across 10 fishing zones, each with a set total allowable catch (TAC, Schiel, 1992). Effort is restricted through the regulation of a snorkel-only fishery.

The South African abalone fishery began in 1949 and is reliant on one species, *Haliotis midae*. The annual harvest fluctuated between 500 t and 1500 t for many years, but in 1965 reached 2800 t (Tarr, 1992). From this point on, the catch (and catch per unit effort) rapidly declined, due to overfishing. In 1968, a production quota of 2316 t was imposed but had no effect (i.e. it was not reached). The quota was decreased annually and first limited the catch (at 1362 t) in 1970 (Tarr, 1992). The quota has remained around 600 t since the early 1970's. Since 1994, intense poaching activities, run by organised crime syndicates, have had devastating effects on the fishery (Tarr, 2000). In early 2004, the South African Government was moving towards a moratorium on recreational harvest of abalone and listing it as a vulnerable species.

The two main Haliotid species fished in southern Australian waters are the blacklip (*H. rubra*) and greenlip (*H. laevigata*) abalone. Blacklips are distributed from Rottnest Island, Western Australia (WA) to Coff's Harbour, New South Wales (NSW) as well as the Bass Strait islands and around Tasmania (Shepherd, 1973). Greenlip abalone have a similar western limit (Cape Naturaliste, WA) but their range only extends to Corner Inlet in Victoria, the Bass Strait islands and the north coast of Tasmania (Shepherd, 1973). Greenlip abalone form the majority of the catch in WA, whereas in NSW, Victoria (Vic), Tasmania (Tas) and South Australia (SA), most (or all) of the catch is blacklip. Recent (yr 2003 or 2003-2004 season depending on state) TAC limits for blacklips were 281 t, 1396 t, 2467.5 t, 482 t and 37.4 t for NSW, Vic, Tas, SA and WA, respectively (D. Worthington, pers. comm., www.dpi.vic.gov.au, Anon, 2002, S. Mayfield, pers. comm. A. Hart, pers. comm.). Corresponding figures for greenlips were 0 t, 0 t, 140 t, 353 t and 202.5 t, respectively.

The Tasmanian abalone fishery began in 1963 (Cuthbertson, 1978), after a minimum size limit (127 mm SL) was set the previous year (Prince and Shepherd, 1992). Commercial abalone licenses were introduced in 1965 and the number of divers capped at 120 in 1969 (Anon, 2000). A further 5 licenses, to fish the Furneaux Group only, were granted in 1972. Licenses became transferable in 1974 and annual catches gradually rose to a peak of 4500 t in 1984. The following year an ITQ system was introduced (Prince and Shepherd, 1992), with each quota unit equivalent to 1.1 tonnes of live abalone caught. This equated to TAC of 3806 t. Since then, minimum size limits and the value of each quota has been varied several times. In 2000, the fishery was divided into three zones, then (in 2003) five zones; four for blacklips and one for greenlips (www.dpiwe.tas.gov.au). The value of the catch in 2002 was $115 million (www.dpiwe.tas.gov.au). The corresponding figure for 2003, although not yet released, was significantly depressed by a reduction in demand, especially in east Asia, caused by Severe Acute Respiratory Syndrome (SARS).

1.3 Abalone culture

Research on the culture of abalone began in Japan, with early works describing the larval development and small scale propagation of *H. gigantea* (Murayama, 1935) and *H. discus* (Ino, 1952). During the 1960's, over a dozen government laboratories began programs to develop hatchery systems (Leighton, 2000). The most significant developments arose in the early 1970's, primarily due to the work of Nagahisa Uki and Shōgo Kikuchi. These researchers published a series of works (in Japanese) on the artificial spawning of abalone (*H. discus*, *H. discus hannai* and *H. gigantea*) covering topics such as the effect of temperature and nutrition on broodstock conditioning, ultraviolet (UV) induction of spawning and optimal sperm density for fertilization (summarized in English in Uki and Kikuchi, 1984). A greater understanding of the process of gonad maturation and the ability to spawn broodstock on demand (using UV induction) greatly improved hatchery and nursery production.

The focus of abalone propagation in Japan is on fisheries enhancement rather than captive growout. At present, 34 prefectural research stations produce seed of one or more of four species (two of which have two subspecies; N. Takiguchi, pers. comm.) that are distributed for release by fishing cooperatives. Currently, nearly 30 million seed are released annually (Kawamura, 2003) and the annual harvest (also managed by fishing cooperatives) stands at 2682 t (2002 figure, Gordon and Cook, 2004). A further 200 t is cultured entirely in captivity (Gordon and Cook, 2004).

Attempts to culture abalone in California began in the mid 1960's (Leighton, 2000). While there was some technology transfer from Japan, it was soon apparent that hatchery techniques would have to be tailored to the needs of local species, the red abalone *H. rufescens* being the preferred candidate. This research was conducted by a small number of private companies and government agencies and by the early 1970's one group (California Marine Associates) had succeeded in growing product to market size (Leighton, 2000). Their success was the catalyst

for further research and investment in the industry. At present, there are 11 groups along the Californian coast that are producing or intend to produce commercial quantities of abalone (Leighton, 2000). The only other abalone farm in the USA cultures the introduced Japanese abalone *H. discus hannai* on the Kona coast of Hawaii. As of 2002, production of American abalone farms was 169 t (Gordon and Cook, 2004) with the total value of abalone products at $US 5.7 million (Seavey, 2003).

The heightened interest in abalone research in the USA during the 1970's led to the important discovery that low (5mM) concentrations of hydrogen peroxide (H_2O_2) also induced abalone to spawn (Morse et al., 1977). These authors proposed that one or more products of the decomposition of H_2O_2 (e.g. the hydroperoxy free radical, HOO·, or the peroxy diradical, ·OO·) act on the enzyme system that produces prostaglandin, which in turn initiates spawning. It is thought that UV irradiation of seawater produces similar free radicals, but the donor molecule is ozone (O_3) rather than H_2O_2 (ozone being produced by the photolysis of dissolved oxygen in seawater). While the peroxide method was developed in the USA, not all hatcheries there use it, with several still preferring to use the UV method.

Propagation of abalone in South Korea, China and Taiwan began in the early 1970's. The main species produced in South Korea and northern China is *H. discus hannai*, while in southern China and Taiwan it is *H. diversicolor* (Chen, 1989; Yoo, 1989; Nie, 1992). Hatcheries in all three countries use the UV method to induce spawning. In South Korea, abalone seed are produced for fisheries enhancement (Yoo, 1989), whereas in China and Taiwan they are grown out in captivity, either in subtidal cages, land-based systems or intertidal ponds. China and Taiwan are the two largest producers of cultured abalone with 2002 annual figures at 4500 and 3000 t, respectively (Gordon and Cook, 2004). No recent production figures for South Korea are available.

In the 1980's, proponents of abalone aquaculture in the USA sold their technology to countries such as Mexico and Chile (Viana, 2002). The two farms currently operating in Mexico culture *H. rufescens* and *H. fulgens*, with a combined annual 2002 production of 53 t (Viana, 2002, Gordon and Cook, 2004). Chilean abalone aquaculture (which started in the early 1990's) is based on the introduced species *H. rufescens* (from North America) and *H. discus hannai* (from Japan). Both are c cultured in northern Chile, predominantly in large land-based facilities, whereas in the south only *H. rufescens* is grown in subtidal cages (Flores-Aguilar, 2003). There are currently 8 farms in the north and 12 farms in the south, with a combined annual (2002) production of 150 t (Flores-Aguilar, 2003, Gordon and Cook, 2004).

Study on the culture of *H. midae* in South Africa began in 1981 (Genade et al., 1988) but it was not until the early 1990's that a systematic research program was initiated (Sales and Britz, 2001). The industry now consists of 12 farms, the majority being land based, with at least one involved in reseeding (Sales and Britz, 2001). Hatcheries typically use H_2O_2 to induce spawning, with one farm having a strict protocol of spawning broodstock every 3 months (regardless of larval needs) to ensure a predictable spawning response when required (M. Miles, pers. comm.). Total production of South African cultured abalone in 2002 was 450 t (Gordon and Cook, 2004) with projections for 2004 in the vicinity of 800 t (Sales and Britz, 2001).

Spawning trials (using H_2O_2) of blackfoot abalone (*H. iris* or paua) in New Zealand began in 1980 and by the late 1980's two land based paua farms had established (Tong and Moss, 1992). The number of farms has since grown to 22 (Kabir, 2001) with the primary aim of most operations being pearl rather than meat production. Juvenile paua are 'seeded' with nuclei which are gradually covered in nacre produced by the mantle tissue. The resultant 'mabe' or half pearls are removed 2-3 years after nucleation and used to produce jewelry such as earrings and necklaces.

Culture experiments on Australian abalone (*H. rubra* and *H. laevigata*) began in Tasmania and Port Lincoln, South Australia in the early 1980's (Sumner and Grant, 1981; Hone and Fleming, 1998). By 1990, there were approximately 10 small farms operating and in 1993 a national program of industry consultation and strategic research was initiated. This collaboration between industry and research providers has resulted in the development of artificial diets, improvements in larval settlement and juvenile growth and genetic improvement programs. Current (2002) annual production from the nearly 40 abalone farms in Australia is 162 t (Gordon and Cook, 2004). Most of these are land based and either produce their own seed or buy in from elsewhere. Hatchery and nursery systems are similar between farms (i.e. use UV induction and conventional settlement plates) but growout systems vary markedly. Those in use today include: raceways (up to 1 m deep) with concrete blocks or tiles for hides; pipe systems, which use hundreds of lengths of 150 mm diameter PVC pipe; maze tanks, 1.2 x 3.0 m moulded polypropylene tubs with a series of straights and 180º turns; and slab tanks, concrete slabs with a low perimeter wall, shallow water (4-5 cm) and occasional flushing from a "tipper". Species differences in behaviour mean that not all systems are used to culture both species. Blacklip abalone are more cryptic than greenlips and so are not suited to maze or slab tanks, neither of which offer any shelter. Other, less frequently used systems for abalone culture in Australia include subtidal cages and barrels and also large cargo vessels fitted out with maze tanks. The latter method has the advantage of being able to follow a water mass of a desired temperature or move to avoid disease or pollution.

Despite there being many abalone farms in Australia, consistent production of seed is still a problem. Almost all hatcheries collect adults from the wild, but spatial and temporal variations in the availability of gravid broodstock and/or the stresses of capture and transport often compromise induction success. Hence, a reliable means of larval supply is vital for the expansion of the industry.

1.4 Reproductive biology and early life history

Artificial control of gonad maturation (i.e. conditioning) of abalone can be achieved through the provision of a favourable physico-chemical environment. This includes a stable water temperature that optimises gonad growth, high levels of dissolved oxygen, low levels of nitrates and ammonia and a pH of 7.5–8.5. Nutrition is also important during this process and broodstock should be fed a high quality diet in amounts slightly in excess of their needs (Uki and Kikuchi, 1982).

Temperature is the main factor influencing the rate of gonad development in most species of abalone. Its effect is cumulative above a certain threshold temperature that varies between species. Kikuchi and Uki (1974b) were the first to record this phenomenon, and named the threshold temperature the biological zero point (BZP). By subtracting the BZP from the daily water temperature and summing this figure over the culture time (in days) they were able to describe the Effective Accumulative Temperature in degree days (EATºC-days) for gonad conditioning of two Japanese abalone species. At present, there is only one account of the EAT for conditioning of southern hemisphere abalones, that of Kabir (2001) on *H. australis* and *H. iris*. Determination of the BZP for gonad development and the optimal EAT for spawning of blacklip and greenlip abalone would be of considerable benefit to hatcheries as they could implement a conditioning regime that resulted in consistently high spawning performance, both in terms of the spawning rate and number of gametes produced. This in turn would greatly improve hatchery efficiency.

The lecithotrophic larval stage of abalone demands that the egg contains sufficient energy reserves to last for several days. These reserves consist primarily of lipid (Moran and Manahan, 2003) which, combined with high fecundity of abalone, means that oogenesis is an energetically demanding process. Fatty acids (FA) are perhaps the most important lipids as they are the major component of cell membranes and in some cases are hormone precursors. Haliotids cannot

synthesize all the FA required for normal cellular function and growth (Uki et al., 1986), and rely on dietary sources of these essential fatty acids (EFA) to fulfill their requirements. Restricting the intake, either through reduced feed rations or provision of feeds low in EFA, results in suboptimal growth of abalone (Uki et al., 1986; Floreto et al., 1996; Mai et al., 1996; Dunstan et al., 2000).

In several countries where abalone are farmed, economic and/or ecological concerns regarding the collection of macroalgae for abalone culture have led to the development of formulated feeds. These feeds are usually composed of a mixture of animal and plant products, and as such have very different FA profiles to those of macroalgae. As yet, the effect of formulated feeds on the lipid and FA profile of somatic and gonadal tissues from blacklip and greenlip abalone (the main species cultured in Australia) has not been examined. Identifying the FA important to gonad development may aid in formulating more suitable broodstock feeds for these species.

Diet is not the only factor influencing the FA composition of marine invertebrates. Freezing points of FA are relatively high and inversely related to the degree of unsaturation. Hence, low temperatures may lead to saturated FA freezing, thus reducing membrane fluidity and disrupting membrane function. Several aquatic invertebrates are able to compensate for this by increasing the proportion of unsaturated FA in cell membranes at low temperatures (Lehti-Koivunen and Kivivuori, 1998; Hall et al., 2002), a phenomenon known as homeoviscous adaptation (Sinensky, 1974). The capacity of abalone to alter their FA profile in response to different temperatures has not been studied. Separating the potential effect of temperature from that of diet may assist in feed formulation.

Given the importance of the fishery to the Tasmanian economy, significant effort has been directed towards monitoring the status of the stocks during the last two decades. Reports generated from surveys have improved our knowledge of growth rates and fecundity of greenlip, and in particular, blacklip abalone. However, one area that has received little attention is the early life history of these species.

Since abalone are broadcast spawners, a reduction in density of mature abalone through fishing or other events has serious implications on fertilization success. In greenlip abalone, separation distances of just two metres can result in a 45% decrease in fertilization success compared to that of adjacent animals (Babcock and Keesing, 1999). In extreme cases, animal density may be so low that by the time sperm reaches an egg, fertilization is highly unlikely, a phenomenon known as the Allee effect (Allee, 1931). With the exception of the work of Babcock and Keesing (1999) on greenlip abalone, there is scant information on the fertilization biology of the two most commercially important haliotids in Australia. Factors requiring clarification for one or both species include the effects of sperm density, gamete contact time and gamete age on fertilization success. Descriptions of sperm morphology are also lacking for blacklip abalone.

Temperature also has a major influence on the early life history of abalone. It dictates the rate of larval development and in so doing affects the duration of the dispersal window (i.e. the interval between hatchout and metamorphic competence). The rate of larval development in abalone is not simply multiplicative (i.e. does not proceed twice as fast if one doubles the temperature) rather, there is a critical minimum temperature (the BZP) below which larval development is arrested (Seki and Kan-no, 1977). Furthermore, the appearance of each stage corresponds to the cumulative difference between water temperature and the BZP (i.e. the EAT). The EAT for each stage (expressed in EATºC-h) is constant between the BZP and the upper thermal limit of the species and provides a means of predicting its appearance when the timing of fertilization and water temperature are known. The ability to predict the duration of the dispersal window from water temperature is necessary to develop models of larval transport for haliotids. Knowledge of the EAT for hatchout and settlement would also enable abalone hatchery managers to control the onset of these stages by manipulating water temperature.

1.5 Objectives of study

The broad aim of this study was to address deficiencies in our knowledge of the reproductive processes of blacklip (*H. rubra*) and greenlip (*H. laevigata*) abalone in order to improve their hatchery production and aid in the management of their fisheries. The hypotheses to be tested were:

- Do these species conform to the BZP model of reproductive development?
- Does temperature and/or conditioning interval influence spawning success?
- Does temperature and/or spawning status affect the lipid and FA composition of somatic and gonadal tissues?
- Does sperm density and/or contact time influence fertilization success?
- Does temperature affect embryonic and larval development?

Each topic is addressed in a separate chapter (each of which contains several sub-topics) outlined below:

Chapter 2 – describes the effect of water temperature on gonad development of both sexes of blacklip and greenlip abalone. Several indices were used to quantify development: descriptors of gross structure include the Visual Gonad Index (VGI) and Estimate of Gonad Volume (EGV). The latter index was divided by shucked animal weight to provide a size-independent measure of gonad development, the Modified Gonad Bulk Index (MGBI). Oocyte diameter ratio (ODR), standardized oocyte diameter, oocyte area and oocyte volume (based on an ellipsoid) were used as descriptors of ovarian microstructure. For each sex and species, the rate of increase in the VGI, MGBI and oocyte volume (females only) of animals held at different temperatures were used to estimate the BZP.

Chapter 3 – examines the effect of two temperatures and five conditioning intervals on the spawning success (in terms of the percentage of spawners, repeat spawners and gamete production) of both sexes of blacklip and greenlip abalone over two conditioning cycles. This information will benefit Australian abalone hatcheries as it identifies the optimal temperature and EAT interval for repeated spawnings of these species.

Chapter 4 – documents the effect of temperature and spawning status on the lipid and FA composition of blacklip and greenlip abalone fed a formulated feed. Foot, digestive gland and gonad samples were analyzed in order to determine where particular FA are synthesized, stored or metabolized. Tissue FA profiles from abalone fed a formulated feed are compared to those from macroalgal feeding trials to determine if the formulated feed can be further improved.

Chapter 5 – investigates the interaction between sperm density and gamete contact time on the fertilization success of blacklip and greenlip abalone. These data were then logit transformed in order to facilitate inter-specific comparisons in fertilisation success across a range of gamete contact times and sperm densities A description of sperm morphology for both species is also provided.

Chapter 6 – reports on the effect of temperature on the larval development of blacklip and greenlip abalone. Stages examined include the first and second polar body release, first and second cell division, hatchout, completion of the velum, torsion and the formation of the fourth tubule on the cephalic tentacle (i.e. metamorphic competence). The duration of the dispersal window (i.e. between hatchout and metamorphic competence) is important to early life history models for Haliotids.

Chapter 7 – summarizes and integrates the main findings of preceding chapters.

1.6 Notes on this study

Each of the five research chapters included in this thesis contain a manuscript written in the format of the journals Aquaculture, Aquaculture Research, Invertebrate Reproduction and Development or the Journal of Shellfish Research. Using this style has necessitated some overlap in introduction, methods and reference sections of the research chapters. Publications are co-authored by the candidate's supervisor or co-supervisor/s in recognition of intellectual and technical contribution given. The status of each publication derived from the study is given below.

Chapter 2 – Temperature effects on the dynamics of gonad and oocyte development in captive wild-caught blacklip (*Haliotis rubra*) and greenlip (*H. laevigata*) abalone. Invertebrate Reproduction and Development. 45. 185-196.

Chapter 3 – 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.

Chapter 4 – Lipid and fatty acid composition of pre- and post-spawning blacklip (*Haliotis rubra*) and greenlip (*H. laevigata*) abalone conditioned at two temperatures on a formulated feed. Aquaculture, 242. 297-311.

Chapter 5 – The effects of sperm density and gamete contact time on the fertilisation success of blacklip (*Haliotis rubra*; Leach, 1814) and greenlip (*H. laevigata*; Donovan, 1808) Journal of Shellfish Research. In press.

Chapter 6 – Temperature effects on embryonic and larval development of blacklip (*Haliotis rubra*) and greenlip (*H. laevigata*) abalone. Invertebrate Reproduction and Development. 45. 197-203.

The research described in Chapters 2 and 3 was part of a larger study funded by the Australian Fisheries Research and Development Corporation managed through the Abalone Aquaculture Subprogram (project number 2000/204).

1.7 Glossary

Absolute oocyte area (OA_{abs}): the area inside the perimeter of the oocyte.

Biological Zero Point (BZP): the temperature below which gonadal (or larval) development is arrested.

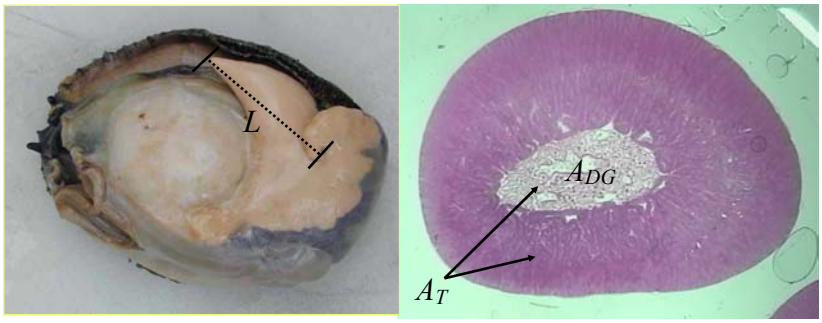
Effective Accumulative Temperature (EAT): the sum of the difference between the culture temperature and the BZP, calculated daily for gonad development and hourly for larval development.

Estimate of Gonad Volume (EGV): calculated from the length of the conical appendage (L) and the cross-sectional areas of the conical appendage (A_T) and the digestive gland (A_{DG}) according to the formula:

$$EGV = \frac{A_T L}{6}\left(8 - \left(\sqrt{\frac{A_{DG}}{A_T}} + 1\right)^3\right)$$

derived by Lleonart (1992).

Figure 1.1 Explanatory diagram showing the length (L) and the cross-sectional areas of the conical appendage (A_T) and the digestive gland (A_{DG}).



Maximum diameter ($max_{Ø}$): the greatest uninterrupted distance inside the perimeter of the oocyte.

Maximum radius (max_r): = $max_{Ø}/2$.

Mean radius ($mean_r$): = min_r + max_r/2.

Minimum diameter ($min_Ø$): the greatest distance perpendicular to the $max_Ø$.

Minimum radius (min_r): = $min_Ø$/2.

Modified Gonad Bulk Index (MGBI): a size independent estimate of gonad bulk, calculated according to the formula: MGBI = EGV/ WW where WW = the shucked wet weight of the animal.

Standardized diameter ($stand_Ø$): = $min_Ø$ + $max_Ø$/2.

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Chapter 2 Temperature effects on the dynamics of gonad and oocyte development in captive wild-caught blacklip (*Haliotis rubra*) and greenlip (*H. laevigata*) abalone

2.1 Abstract

Wild-caught blacklip (*Haliotis rubra*) and greenlip (*H. laevigata*) abalone were held from spent condition at 12ºC, 14ºC, 16ºC or 18ºC and routinely sampled to examine gonad development. Descriptors of gross structure included the Visual Gonad Index (VGI) and the Modified Gonad Bulk Index (MGBI). Oocyte Diameter Ratio (ODR) and oocyte volume (based on an ellipsoid) were used as descriptors of ovarian microstructure. For each species, the rate of increase in the VGI, MGBI and oocyte volume of animals held at different temperatures were used to estimate the Biological Zero Point (BZP), the critical temperature below which no development occurs. BZP estimates derived from the daily increase in VGI and oocyte volume were similar (7.8ºC and 7.6ºC for blacklip abalone; 6.9ºC and 6.8ºC for greenlip abalone, respectively), but those based on the increase in MGBI were up to 1.8ºC lower (6.0ºC and 5.7ºC, for blacklip and greenlip abalone, respectively). The mean MGBI, in terms of gonad volume per gram of shucked animal weight, ranged from 5–68 mm^3g^{-1} and 5–58 mm^3g^{-1} for blacklip and greenlip abalone, respectively. The ODR indicated that oocyte shape was highly variable in oocytes < 90µm diameter in both species. Above 90µm, ODR values increased proportionally with oocyte size, indicating a transition in shape from elliptical to round. Ranges for mean oocyte volume for blacklip and greenlip abalone were 0.15–1.4 x 10^6 μm^3 and 0.02–1.83 x 10^6 μm^3, respectively. The pattern of oocyte growth relative to temperature for both species is illustrated using tables of standardized residuals. Determination of the BZP for blacklip and greenlip abalone enables the calculation of the Effective Accumulative Temperature (EAT; the cumulative difference between the water temperature and the BZP, calculated daily) for gamete maturation of these species. This in turn facilitates predictive and deductive estimates of the completion of this process (when water temperature is known) in either natural or artificial (i.e. culture) environments.

2.2 Introduction

Blacklip (*Haliotis rubra*) and greenlip (*H. laevigata*) abalone are herbivorous marine gastropods inhabiting reefs and boulder fields in waters off southern Australia. Commercial harvesting of these species began in the late 1960's and studies on their reproductive biology were initiated soon after (Harrison and Grant, 1971; Shepherd and Laws, 1974). Shepherd and Laws (1974) demonstrated that the reproductive cycle of these species in South Australia varied both annually and geographically, with varying degrees of seasonality in spawning. Subsequent studies on stocks from Victoria (McShane et al., 1986), Tasmania (L. Gurney, pers. comm.) and Western Australia (Wells and Mulvay, 1992) have also reported seasonal peaks in spawning, but as yet no direct link between gonad development and environmental variables has been established in these species.

Kikuchi and Uki (1974a,b) first quantified the effect of temperature on sexual maturation of haliotids. They monitored the increase in the Visual Gonad Index (VGI) of *H. discus hannai* and *H. discus discus* at different water temperatures. The VGI is a non-invasive, semi-quantitative means of assessing gonad bulk in abalone and consists of four categories (0–3), describing successive changes in the size and shape of the gonad. At temperatures within the normal range for each species (and where food was provided in excess), there were positive linear relationships between the VGI (when the mean VGI ≤ 2.5) and conditioning time. The rate of increase in VGI was proportional to water temperature, and when each value was plotted against water temperature, the zero value for VGI could be interpolated. This gave an estimate of the Biological Zero Point (BZP), the temperature below which gonad development is arrested. By subtracting the BZP from the daily water temperature and summing this figure over the conditioning time (in days), Kikuchi and Uki (1974a,b) were able to describe the Effective Accumulative Temperature (EAT, expressed as EATºC-days) for completion of gametogenesis and spawning. At present, there is only one account of the EAT for gamete maturation of southern hemisphere abalone (Kabir, 2001; for *H. australis* and *H. iris*).

Reproductive development in abalone may also be quantified by gonad indices and oocyte size. Gonad indices are based on cross-sectional measurements of the conical appendage (i.e. the gonad-digestive gland complex) and vary in their complexity and accuracy (see Hahn, 1989 for a review). The Modified Gonad Bulk Index (MGBI) of Tutschulte and Connell (1981) uses linear measurements from both the exterior and interior (i.e. the dimensions of each tissue in cross-section) of the conical appendage to produce the Estimate of Gonad Volume (EGV). The EGV is then divided by the shucked wet weight of the animal to yield the MGBI. Lleonart (1992) showed that the EGV could be derived using area rather than linear measurements. Furthermore, the formula given by Lleonart (1992) uses fewer terms than that of Tutschulte and Connell (1981) making it easier to compute.

Measurement of oocyte size as a means of describing reproductive development in marine organisms was first undertaken by Thompson (1915 cited by Clark, 1934). However, oocyte size frequency polygons are often difficult to interpret and are not amenable to significance testing. Grant and Tyler (1983a,b) reviewed the various means of describing and analyzing reproductive patterns in marine invertebrates. They promoted contingency table analysis as a means of determining statistically significant differences in oocyte size frequency data, and tables of standardized residuals to show the location of these differences, with positive residuals indicating a greater than expected frequency of oocytes in that size class, and negative residuals a lower than expected frequency of oocytes. The use of contingency table analysis and tables of standardized residuals has become common in studies dealing with changes in oocyte size (expressed as diameter, area or volume) frequency in abalone held under artificial (e.g. constant temperature) conditions (Lleonart, 1992; Hahn, 1994; Moss, 1998; Kabir, 2001). However, this method has only been used once for wild abalone (Kabir, 2001), despite the large number of studies on reproduction in wild haliotids.

The aim of this study was to first, describe gonad and oocyte development of blacklip and greenlip abalone held at four different temperatures (12, 14, 16 or 18ºC) using several different indices (VGI, MGBI and oocyte size frequency) and second, to use the daily rate of change of these indices to calculate the BZP for gonad development of both species. Knowledge of BZP enables the calculation of the EAT for gamete maturation of these species (when the daily temperature and time interval are known). This allows comparisons of the gametogenic cycle between species, between wild and domesticated (i.e. cultured) stocks and also between populations with different thermal histories. An alternative means of calculating oocyte volume and an improved contingency table format is also presented.

2.3 Methods

2.3.1 Collection and inspection of animals

Blacklip and greenlip abalone were collected from West End, Settlement Point and Roydon Island (Furneaux group, north east Tasmania) on 23 November 2000 and 27 April 2001. Animals were transferred to the Tasmanian Aquaculture and Fisheries Institute, Marine Research Laboratories and held in 600 L tanks supplied with ambient temperature seawater until examination and allocation to experimental treatments. Prior to the start of the experiment (10 May 2001), animals were measured, weighed, tagged and VGI assessed according to the following criteria: 0 = sex indistinguishable; 1 = sex distinguishable, thin gonad with pointed tip; 2 = gonad partially enlarged with pointed tip; 3 = gonad swollen with rounded tip. In both species, the size range for experimental animals was 100-130 mm shell length (SL). Blacklip abalone ranged from 143–334 g (mean = 218 g) and greenlip abalone from 107–323 g (mean = 210 g). The majority of animals of both species had VGI scores of 0. Animals with VGI values of 2 or 3 were induced to spawn using heated UV-irradiated seawater (Grubert and Ritar, 2002). Only animals that spawned or had 0 scores when first examined were used

in the experiment. Of these, five to six animals of each sex and species were killed immediately post spawning and the gonad processed as detailed below. Animals generally appeared healthy, but the shells of some greenlips had small colonies of the spionid polychaete mudworm *Boccardia knoxi*.

2.3.2 Experimental design

Abalone of each sex and species were held in separate 150 L fibreglass tanks. Within each sex and species group, 36 animals were randomly assigned to each of four experimental temperatures (12, 14, 16 or 18ºC; the range being determined by the capacity of the heat/chill units) with two tanks per temperature treatment (2 x 2 x 4 x 2 = 32 tanks total). The time interval for sampling was based on putative BZP figures (for the purpose of setting sampling dates only) of 7.0ºC for blacklip abalone and 8.0ºC for greenlip abalone and an EAT interval of 400ºC-days for both species. For blacklip abalone, the number of days between each sampling (n = the number of samplings at each temperature) was 80 days at 12ºC (n = 3), 57 days at 14ºC (n = 4), 44 days at 16ºC (n = 6) and 36 days at 18ºC (n = 6). Corresponding values for greenlip abalone were 100 days at 12ºC (n = 3), 67 days at 14ºC (n = 4), 50 days at 16ºC (n = 6) and 40 days at 18ºC (n = 60). At these times, VGI was determined in 4-6 animals in each treatment group (selected at random from the two tanks) and the mean VGI plotted against culture time for each temperature treatment. The slopes of these lines were then plotted against water temperature and the x-intercept (and associated confidence limits) determined using inverse prediction (Zar, 1996).

2.3.3 Husbandry and monitoring

Each experimental temperature was maintained using a 10kW heat-chill unit, with flow rate to each tank set at 1.5 L min^{-1}. Temperature was recorded using StowAway TidbiT temperature loggers (Onset Computer Corporation, Massachusetts) in each system. Animals were fed daily to satiation on a broodstock conditioning diet (Adam and Amos Abalone Foods Pty Ltd, South

Australia). Photoperiod was maintained at 12L:12D starting at 06:00h and light intensity at the bottom of the tanks (when filled) was 90–100 Lux.

2.3.4 Histology

Following determination of the VGI, abalones were shucked, weighed and the distance from the anterior (i.e. tip) of the conical appendage to the apex of the visceral spire was measured and the mid-point calculated (similar to that illustrated in Ault, 1984 except that posterior measurement was to the apex of visceral spire not the base of the conical appendage). A transverse cut was made at the mid-point and the anterior section fixed in Formaldehyde Acetic Acid Calcium Chloride (FAACC). Gonad samples were embedded in wax, sectioned transversely at 6 µm and stained using Mayer's Haematoxylin and Young's Eosin.

2.3.5 Calculation of the Modified Gonad Bulk Index and measurement of oocytes

Images of conical appendage sections were captured using Leica IM50 software. The cross-sectional areas of the conical appendage (A_T), and digestive gland (A_{DG}) were calculated using SigmaScan Pro 5.0. The two area measurements and the length (L) of the conical appendage were used to derive the EGV according to Lleonart's (1992) formula:

$$EGV = \frac{A_T L}{6}\left(8 - \left(\sqrt{\frac{A_{DG}}{A_T}} + 1\right)^3\right)$$

An assumption of the EGV is that the conical appendage consists of two cones; the smaller, inner cone is the digestive gland, and the remaining volume in the larger, outer cone is the gonad (see Ault, 1984 for explanatory diagram). Given that the volume of a right circular cone equals 1/3 base area x length, it is possible to estimate gonad volume from the total area of the conical appendage minus the area occupied by the digestive gland. The EGV and shucked wet weight (WW) of each animal were then used to calculate its MGBI according to the formula MGBI = EGV/WW.

For the measurement of oocytes, one hundred oocytes with a distinct nucleus (including polygonal oocytes) were traced for each female. As a precaution against heterogeneity of development within the ovary, the oocytes were measured along a transect through the center of the section. The image analysis software was set to record different area and diameter (Ø) measurements defined as follows: absolute oocyte area (OA_{abs}), the area inside the perimeter of the oocyte; maximum diameter ($max_{Ø}$), the greatest uninterrupted distance inside the perimeter of the oocyte and the minimum diameter ($min_{Ø}$), the greatest distance perpendicular to the $max_{Ø}$. Other measurements derived from these included the standardized diameter ($stand_{Ø} = (min_{Ø} + max_{Ø})/2$), minimum radius ($min_r = min_{Ø}/2$), maximum radius ($max_r = max_{Ø}/2$) and mean radius ($mean_r = (min_r + max_r)/2$).

Prior to calculating oocyte volume, it was necessary to establish which of the two estimates (spherical volume, $SV = 4/3.\pi.(mean_r)^3$; or ellipsoid volume, $EV = 4/3.\pi.((min_r)^2.max_r)$ was the most appropriate to use. The first step in this process was to determine if oocyte shape varied, as SV estimates are most accurate when oocytes are round. This was achieved by calculating the oocyte diameter ratio ($ODR = min_{Ø}/max_{Ø}$) of 100 oocytes from 10 randomly selected females of each species and plotting these values against $min_{Ø}$. The next step was to ascertain whether estimated area ($OA_{est} = \pi.min_r.max_r$) approximated the absolute area (OA_{abs}) as the two radii used to generate OA_{est} would also be used to calculate oocyte volume. Estimated oocyte area was calculated using the formula for the area of an ellipse, rather than that for a circle as the former takes into account variable radii, whereas the latter does not. In each species, values of OA_{abs} and OA_{est} were calculated for each of the oocytes in the data sets used above, plotted against $min_{Ø}$ and a curve fitted. Regression analyses between $min_{Ø}$, $\sqrt{OA_{abs}}$ and $\sqrt{OA_{est}}$ were run to determine the residual mean square ($MS_{residual}$) for each relationship, $MS_{residual}$ being the best criterion of fit when dealing with non-linear data (Quinn and Keogh, 2002). Correlation analyses were run on $\sqrt{OA_{abs}}$ versus $\sqrt{OA_{est}}$ for each species to establish if OA_{est} approximated OA_{abs}. The final

step in the validation process was to compare and contrast oocyte volume estimates derived using different volume formulae (SV or EV). Both SV and EV were calculated from the same data sets as used previously, plotted against $min_{Ø}$ and a curve fitted. Estimates of SV and EV were cube root transformed and the $MS_{residual}$ determined from the regressions between each variable and $min_{Ø}$, with a low $MS_{residual}$ indicating a better fit and less variability.

Individual oocytes were not staged during this study and so we were not able to determine if potential changes in shape correlated with particular oocyte stages. However, a data set (based on 7218 oocyte observations) on stage and size frequency of oocytes from wild-caught blacklip abalone from southern Tasmania (L. Gurney, unpublished data) enabled a comparison of changes in oocyte shape and stage relative to $min_{Ø}$.

2.3.6 Contingency table analysis

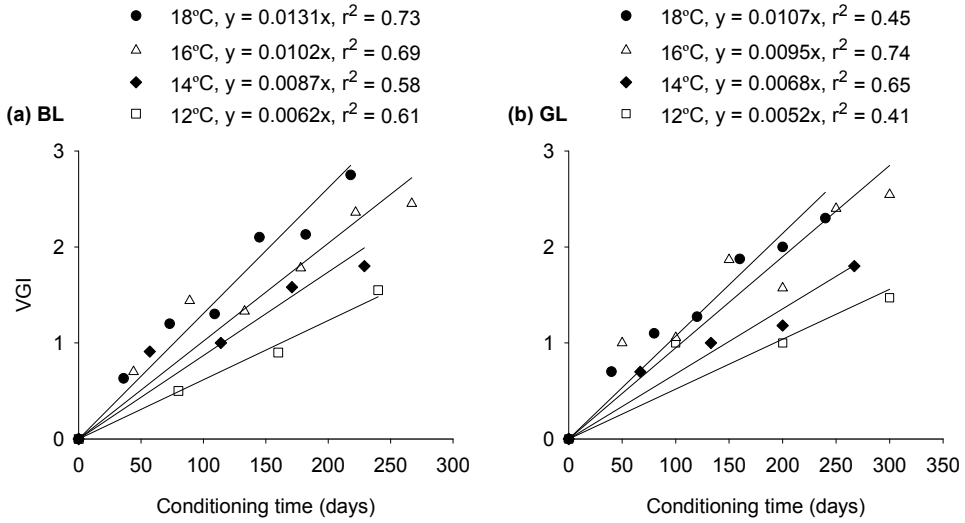
Oocyte size frequency data (derived from 100 oocyte measurements per ovary) for all females at each sampling time were used to construct a (R x C) contingency table, where R is the number of sampling times and C is the number of oocyte size classes (Grant and Tyler, 1983b). Given that all females at each temperature and each sampling time were held under similar conditions, we assumed that they were at the same stage of development when sampled (this was later confirmed by running ANOVA on the oocyte volume data). Thus, the expected frequency (e_{ij}) at each point was calculated as $e_{ij} = (R_i \times C_j)/n$, where R_i is the total number of oocytes in the i^{th} size class summed over all individuals, C_j is the total number of oocytes measured at the j^{th} sampling point and n is the total number of oocytes measured. Observed (o_{ij}) and expected oocyte volume frequencies were used to calculate the χ^2 statistic, according to the formula $\chi^2 = \sum((o_{ij} - eij)^2/(e_{ij}))$, with $(r-1)(c-1)$ degrees of freedom. Tables of standardized residuals for each temperature group were generated by dividing the residual r_{ij} (where $r_{ij} = (o_{ij} - eij)/(e_{ij})^{0.5}$) by the expected variance v_{ij} (where $v_{ij} = (1-(r_i/n)) \times (1-(c_j/n))$) for all combinations of conditioning interval and size class.

2.4 Results

2.4.1 Increase in VGI and MGBI relative to temperature and conditioning interval

Within each species and temperature group, the increase in VGI relative to conditioning time was not significantly different between males and females (See Appendix 1), and VGI data for both sexes were pooled. The mean VGI increased linearly during conditioning in both species (r^2 = 0.58–0.73 and 0.41–0.74 for blacklips and greenlips, respectively) with the rate of change in VGI proportional to temperature (Figs 2.1a and 2.1b).

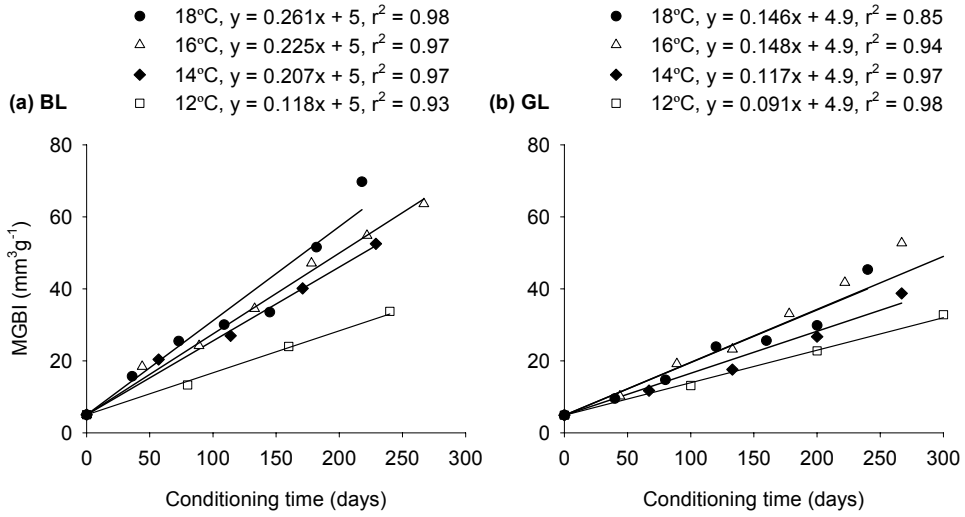
Figure 2.1 Increase in mean Visual Gonad Index (VGI) score relative to conditioning time and culture temperature in blacklip (BL, a) and greenlip (GL, b) abalone. Data for males and females within species were pooled.



The increase in MGBI relative to conditioning time was not significantly different between male and female blacklips or greenlips except for those greenlips held at 16ºC and 18ºC, where males matured faster than females (see Appendix 2). Despite this (and in order to maximize sample size), sex was pooled within species for this analysis. The mean MGBI increased linearly during conditioning in both species (Fig. 2.2) with values ranging from 5–68 mm^3g^{-1} and 5–58 mm^3g^{-1}

for blacklips and greenlips, respectively. There was no relationship between MGBI and shucked weight for blacklip ($r^2 = 0.02$) or greenlip abalone ($r^2 = 0.01$) confirming that MGBI is a size independent measure of gonad bulk in these species.

Figure 2.2 Increase in Modified Gonad Bulk Index (MGBI) relative to conditioning time and culture temperature in blacklip (BL, a) and greenlip (GL, b) abalone. Lines for the greenlip 16ºC and 18ºC treatments overlap. Data for males and females within species were pooled.



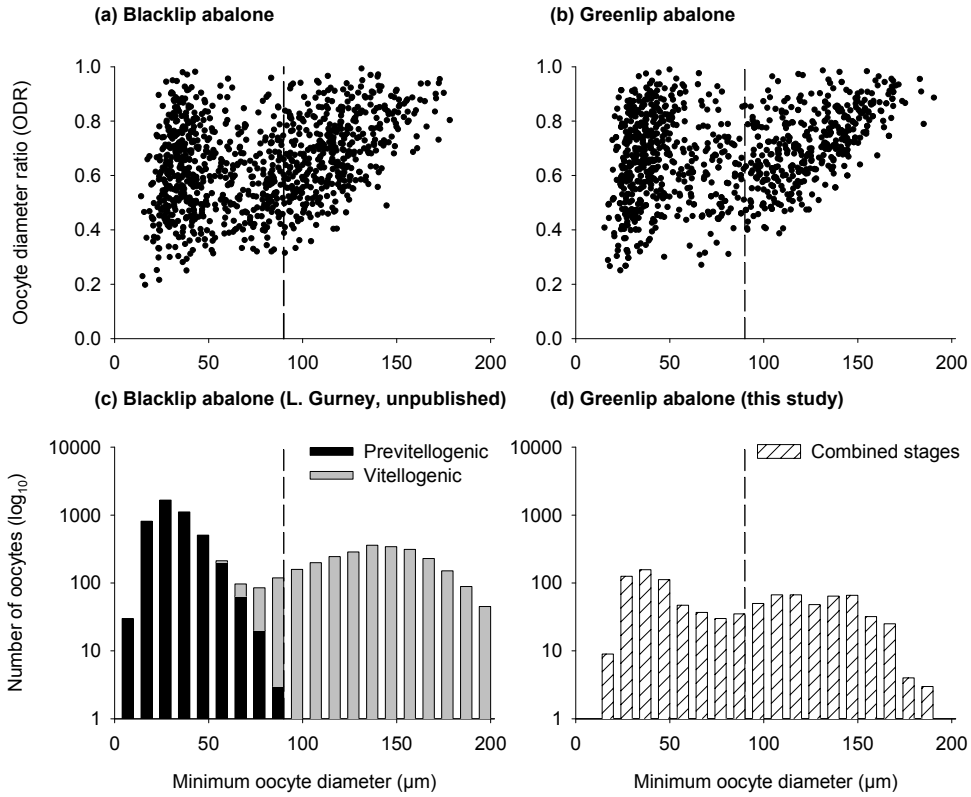
2.4.2 Increase in oocyte size relative to temperature and conditioning interval

The increase in oocyte standardized diameter ($stand_{Ø}$) and absolute oocyte area (OA_{abs}) was positively correlated with temperature and conditioning time in both species. Mean values of the $stand_{Ø}$ and OA_{abs} for blacklips ranged from 59–143 µm and 34–143 x 10^2 µm^2, respectively. Corresponding values for greenlips were 51–160 µm and 27–172 x 10^2 µm^2, respectively.

The oocyte diameter ratio (ODR) of oocytes from both species was highly variable in oocytes with a $min_{Ø}$ <90 µm (Figs 2.3a-b). Above 90 µm, ODR increased proportionally with $min_{Ø}$, indicating a transition in oocyte shape from elliptical to round with increasing oocyte size. From Fig. 2.3c, it can be seen that

100% vitellogenesis occurs in blacklip oocytes >90 µm (L. Gurney, unpublished data), while Fig. 2.3d shows that the size frequency histogram for greenlip oocytes (this study) is similar in form.

Figure 2.3 The relationship between minimum oocyte diameter and Oocyte Diameter Ratio (ODR; minimum diameter / maximum diameter) in (a) blacklip and (b) greenlip abalone as well as stage and size frequency of oocytes in (c) blacklip (from L. Gurney, unpublished) and (d) greenlip abalone (this study). Dashed lines indicates minimum oocyte diameter of 90 µm.



The decision to use EV rather than SV to calculate oocyte volume was based on oocyte shape, which in turn dictated which radii values were used. High variability in ODR meant that few oocytes were perfectly round. Hence, oocyte area and volume (SV) would be over-estimated if the mean of the two radii ($mean_r$) was used. By contrast, the high correlation between OA_{est} ($= \pi.min_r.max_r$) and absolute oocyte area (from image analysis software) in both species ($r = 0.993$ and $r = 0.995$ for blacklips and greenlips, respectively) suggests that using min_r

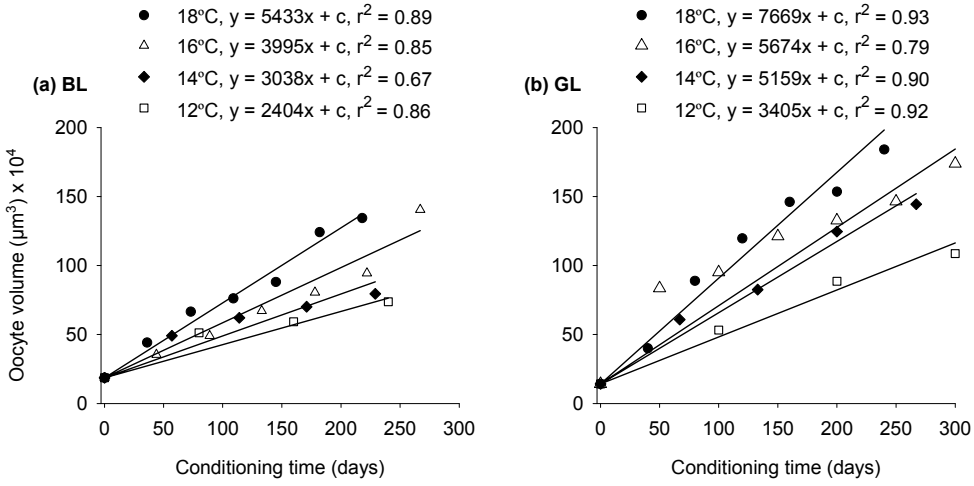
and max_r to calculate EV would produce a more accurate estimate of oocyte volume. The greater variability in SV than EV for a given minimum oocyte diameter (as indicated by the greater $MS_{residual}$ for SV than EV, Table 2.1) is further evidence that EV provides a better estimate of oocyte volume than SV.

Table 2.1 Power functions describing the relationships between minimum oocyte diameter (x) and absolute area (OA_{abs}), estimated area (OA_{est}), spherical volume (SV) and ellipsoid volume (EV) in blacklip and greenlip abalone. The value of the mean square residual ($MS_{residual}$) is proportional to the degree of variability in the data.

	Blacklip		Greenlip	
Variable (y)	Function	$MS_{residual}$	Function	$MS_{residual}$
OA_{abs}	$= 2.381x^{1.824}$	122.3	$= 2.001x^{1.852}$	114.1
OA_{est}	$= 2.220x^{1.864}$	118.5	$= 1.924x^{1.885}$	94.5
SV	$= 3.513x^{2.740}$	165.5	$= 2.656x^{2.782}$	128.5
EV	$= 1.482x^{2.865}$	36.5	$= 1.283x^{2.886}$	30.2

Oocyte volume increased as temperature and conditioning time increased (Figs 2.4a and 2.4b) with mean oocyte volume ranging from 18–140 x 10^4 μm^3 in blacklips and 14–184 x 10^4 μm^3 in greenlips.

Figure 2.4 The relationship between conditioning time (x), culture temperature and oocyte volume (y) in (a) blacklip and (b) greenlip abalone. Values of constant c were 1.86 x 10^4 and 1.42 x 10^4 for blacklip (BL) and greenlip (GL) abalone, respectively.



2.4.3 Estimation of the BZP for gonadal development

The r^2 values for the daily rate of increase in VGI, MGBI and oocyte volume (OV) with temperature were 0.98, 0.90 and 0.99 for blacklips (BL, Figs. 2.5a-c) and 0.99, 0.99 and 0.99 for greenlips (GL, Figs. 2.5d-f), respectively. BZP estimates derived from the VGI and OV were similar for BL (7.8ºC and 7.6ºC) and GL (6.9ºC and 6.8ºC) but were lower for MGBI (BL = 6.0ºC; GL = 5.7ºC). In both species, the most robust estimate of the BZP was derived from the VGI, as evident by the narrower confidence intervals for this variable (Table 2.2).

Table 2.2 Upper and lower 95% confidence intervals (CI) for BZP estimates (in ºC) derived from the Visual Gonad Index (VGI), Modified Gonad Bulk Index (MGBI) and oocyte volume (OV) for blacklip (BL) and greenlip (GL) abalone. Dash indicates slope approximated zero, therefore CI's cannot be calculated.

Measure	Species	BZP	Upper 95% CI	Lower 95% CI
VGI	BL	7.8	9.1	2.6
	GL	6.9	9.9	-1.7
MGBI	BL	6.0	–	–
	GL	5.7	9.3	-9.8
OV	BL	7.6	11.0	-2.2
	GL	6.8	10.6	-4.6

2.4.4 Contingency table analysis of oocyte volume frequency

There were significant differences between the observed and expected frequencies of oocytes in each volume class across the range of EAT conditioning intervals (calculated using the BZP estimates of 7.8ºC and 6.9ºC for blacklips and greenlips, respectively) and temperature treatments for blacklip and greenlip abalone (χ^2 = 254–1281, $p < 0.001$). Tables 2.3 and 2.4 show the standardized residuals for each oocyte volume class against conditioning interval at each experimental temperature for blacklip and greenlip abalone, respectively. The shift in positive residuals from top left to bottom right (i.e. the increase in volume of a cohort of oocytes over time) occurred in a similar fashion in each species and temperature group.

Figure 2.5 The relationship between Visual Gonad Index (VGI), Modified Gonad Bulk Index (MGBI), oocyte volume and culture temperature in blacklip (BL, a–c) and greenlip (GL, d–f) abalone. Linear relationship in 5e did not include the outlier value at 18ºC.

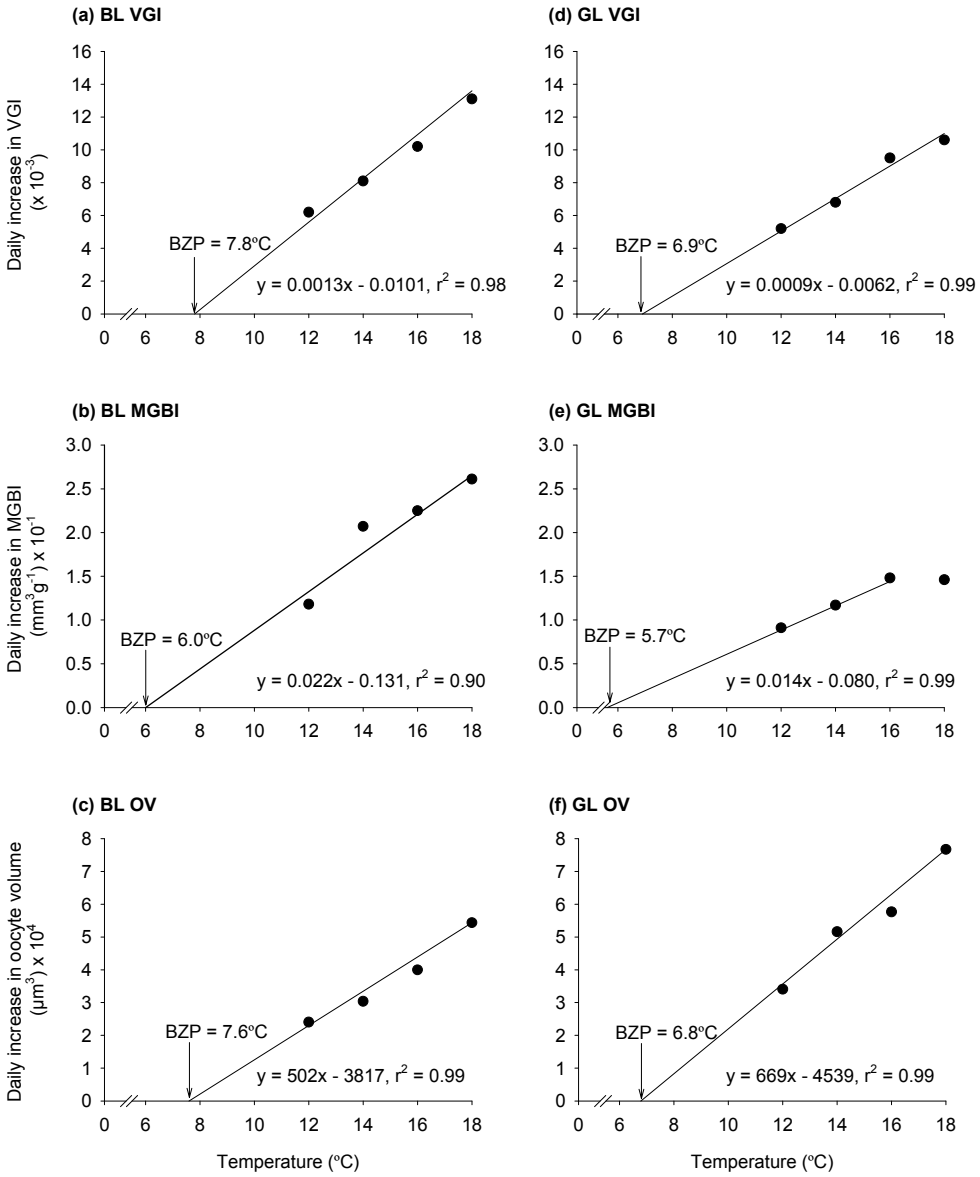


Table 2.3 Contingency table of standardized residuals for frequencies of oocyte volume in female blacklip abalone (n = sample size) at each temperature and conditioning interval. Positive values (in bold) indicate a greater than expected frequency of oocytes in that size class, whereas the negative values indicate a lower than expected frequency.

TºC	EAT (ºC-d)	Oocyte volume class (μm^3 x 10^5)										
		n	0.1	0.2	0.4	0.8	1.6	3.2	6.4	12.8	25.6	51.2
12	0	*3*	**1.5**	**4.8**	**4.1**	**2.8**	**2.1**	**0.1**	-2.8	-6.1	-3.6	-1.3
	330	*3*	**1.0**	**3.4**	**0.3**	-1.8	-1.7	-0.3	0.0	**0.1**	**0.5**	-1.5
	660	*3*	**0.2**	-2.4	-0.7	0.0	-0.1	-0.4	**1.1**	**2.1**	-0.3	-0.2
	990	*5*	-1.5	-3.0	-2.3	-0.9	-0.5	**0.3**	**1.1**	**2.4**	**2.2**	**1.6**
14	0	*3*	**3.1**	**5.6**	**4.3**	**2.0**	-0.4	**0.8**	-2.0	-3.9	-6.1	-1.6
	350	*3*	**0.4**	**2.1**	**0.5**	**1.2**	**0.1**	**0.2**	-0.3	-3.0	-0.7	0.6
	700	*4*	-0.8	-2.2	**0.2**	**1.5**	**0.1**	-0.8	-0.6	**0.8**	**0.7**	**0.4**
	1050	*4*	-0.5	-2.4	-2.1	-2.7	**1.2**	**0.2**	**1.1**	**3.4**	**1.2**	0.0
	1400	*3*	-1.9	-2.0	-2.5	-1.7	-1.3	-0.2	**1.7**	**1.7**	**4.3**	**0.6**
16	0	*3*	**4.9**	**5.5**	**7.7**	**7.4**	**0.2**	-0.4	-1.0	-6.7	-8.3	-3.3
	360	*3*	**1.4**	**4.2**	**4.3**	**3.1**	**2.7**	**1.6**	-0.7	-3.8	-6.7	-2.0
	720	*3*	**2.5**	**2.2**	**4.6**	**1.4**	**2.9**	**0.5**	-1.4	-4.8	-2.4	-2.5
	1080	*3*	**0.7**	**0.5**	**0.6**	**0.3**	**1.8**	0.0	-1.0	0.0	-1.0	-1.5
	1440	*4*	-1.5	-2.0	-2.0	-1.0	-1.3	**1.1**	**2.4**	**4.0**	-0.6	-1.3
	1800	*5*	-2.1	-1.0	-4.1	-3.0	-0.5	0.0	**1.4**	**4.2**	**2.3**	-0.1
	2160	*6*	-3.0	-5.2	-5.6	-4.1	-3.3	-1.9	-0.5	**2.8**	**9.8**	**6.8**
18	0	*3*	**10.0**	**9.5**	**5.5**	**2.0**	**0.9**	**0.9**	0.0	-5.2	-10.0	-3.6
	370	*3*	**1.5**	**2.9**	**3.8**	**5.4**	**3.4**	-0.7	-0.2	-3.3	-6.1	-2.6
	740	*3*	**0.4**	**2.3**	**2.4**	**0.6**	**1.5**	-1.7	**1.6**	**0.5**	-4.7	-1.4
	1110	*3*	-1.9	-1.1	-1.3	**1.6**	**1.8**	**1.9**	**0.8**	**2.8**	-3.4	-1.1
	1480	*4*	-2.2	-0.5	-0.1	-0.8	**0.2**	**1.7**	**2.0**	-0.4	**0.1**	-0.9
	1850	*6*	-2.3	-3.6	-1.9	-2.6	-3.2	-0.5	-1.3	**1.6**	**6.5**	**2.9**
	2220	*6*	-2.3	-4.6	-4.5	-2.8	-1.6	-1.0	-1.6	**2.0**	**8.5**	**3.2**

Table 2.4 Contingency table of standardized residuals for frequencies of oocyte volume in female greenlip abalone (n = sample size) at each temperature and conditioning interval. Positive values (in bold) indicate a greater than expected frequency of oocytes in that size class, whereas the negative values indicate a lower than expected frequency.

TºC	EAT (ºC-d)		Oocyte volume class (μm^3 x 10^5)									
		n	0.1	0.2	0.4	0.8	1.6	3.2	6.4	12.8	25.6	51.2
12	0	*3*	**1.0**	**2.2**	**6.4**	**6.7**	**1.0**	-0.3	-1.8	-5.1	-7.2	-1.8
	500	*4*	**1.2**	**2.0**	**1.4**	-0.3	**1.0**	-0.3	-0.4	-1.6	-1.5	-1.3
	1000	*4*	-0.5	-1.4	-3.5	-2.4	-0.3	**1.4**	**0.8**	**1.6**	**3.4**	**1.4**
	1500	*3*	-1.8	-2.9	-3.8	-3.3	-1.9	-1.0	**1.3**	**5.1**	**4.8**	**1.7**
14	0	*3*	**3.3**	**5.5**	**11.2**	**8.2**	**1.8**	-1.0	-2.1	-5.8	-11.2	-3.2
	470	*4*	**2.5**	**1.8**	**1.7**	**2.7**	**2.4**	**2.6**	**0.2**	-2.4	-5.8	-0.6
	940	*4*	0.0	**0.2**	-1.1	-0.3	**1.8**	**0.5**	**3.8**	**0.4**	-3.2	-0.2
	1410	*5*	-2.1	-1.7	-2.9	-3.2	-1.3	-0.2	-1.7	**1.9**	**6.1**	**1.2**
	1880	*5*	-2.3	-3.7	-5.4	-4.4	-3.4	-1.8	-0.2	**3.7**	**9.2**	**1.7**
16	0	*3*	**4.9**	**5.9**	**15.5**	**13.5**	**5.4**	**1.5**	-0.6	-7.4	-15.7	-4.7
	450	*3*	**5.7**	**5.0**	**3.8**	**2.9**	**0.9**	**0.8**	**2.3**	-4.7	-6.9	**0.3**
	900	*4*	**1.3**	**3.3**	**1.4**	-0.2	**3.5**	**3.6**	**1.5**	-2.9	-2.3	-2.5
	1350	*6*	-1.9	**0.4**	-1.3	-0.3	-1.7	-0.3	-0.3	**2.7**	**0.3**	-0.6
	1800	*6*	-1.7	-2.6	-2.8	-2.6	-1.7	-1.2	**1.1**	**3.3**	**3.7**	-1.2
	2250	*6*	-1.9	-3.5	-4.9	-3.7	-1.0	-0.6	-1.4	**4.2**	**4.7**	**1.3**
	2700	*5*	-2.5	-4.2	-4.9	-4.2	-2.6	-2.4	-1.7	-0.3	**8.4**	**5.9**
18	0	*3*	**5.0**	**5.8**	**12.4**	**11.7**	**3.6**	**0.6**	-1.2	-6.7	-14.1	-3.9
	440	*3*	**3.8**	**6.7**	**3.8**	**5.0**	**3.9**	**3.0**	1.4	-1.8	-11.3	-3.7
	880	*4*	**0.1**	**2.8**	**1.1**	**0.3**	**0.1**	**2.7**	**2.4**	-0.5	-4.1	-1.2
	1320	*4*	-0.8	-2.7	-1.3	-2.7	-0.9	-0.3	**1.9**	**2.9**	**2.1**	-1.6
	1760	*6*	-2.4	-3.2	-4.4	-3.6	-2.0	-1.5	-1.8	**2.3**	**7.9**	**1.2**
	2200	*3*	-1.8	-3.6	-3.7	-4.3	-0.6	-1.8	-0.6	**1.9**	**7.0**	**1.4**
	2640	*3*	-2.2	-3.8	-5.2	-3.7	-2.6	-2.2	-1.9	-0.1	**7.9**	**7.7**

2.5 Discussion

2.5.1 Gonad development

In blacklip and greenlip abalone, estimates of the BZP for gonad development derived from the VGI (BZP_{VGI}) and oocyte volume (BZP_{OV}) were almost identical, while estimates calculated from the MGBI (BZP_{MGBI}) were 1.1–1.8ºC lower. For ease of comparison between species, the BZP values referred to in the remainder of the discussion are the BZP_{VGI} estimates. Estimates of the VGI for *H. rubra* (7.8ºC) and *H. laevigata* (6.9ºC) are similar to that reported for *H. discus hannai* (7.6ºC; Kikuchi and Uki, 1974a) but higher than those for *H. discus discus* (5.3ºC; Kikuchi and Uki, 1974b), *H. australis* and *H. iris* (5.0ºC and 6.2ºC, respectively; Kabir, 2001).

Unlike the situation off the coast of Japan for *H. discus hannai* and *H. discus discus* (Kikuchi and Uki, 1974a,b), minimum monthly water temperatures experienced by blacklip and greenlip abalone in southern Australian waters do not fall below their BZP. Hence, gonad growth is possible throughout the year, with at least part of the population always reproductively mature (Shepherd and Laws, 1974; McShane et al., 1986; Wells and Mulvay, 1992). In theory, water temperatures during summer/autumn (when the differential between the BZP and ambient temperature is greatest) should promote faster gonad growth than in winter/spring. However, in some cases, high summer water temperatures (e.g. > 22ºC) experienced by blacklip and greenlip abalone may stress some (or all) individuals, resulting in gonad development being “deferred” until water temperature fall backs within the preferred range. This, in conjunction with seasonal variations in feed availability and other environmental factors, may explain the presence of mature animals in wild populations at any time of the year.

In mature *H. rubra* and *H. laevigata*, the gonad covers not only the digestive gland, but most of the stomach as well (*pers. obs.*). Indeed, Lleonart (1992) showed that in mature *H. laevigata*, just 24% of the total volume of the gonad resides in the conical appendage. This is in contrast to the findings of Tutschulte and Connell (1981), who stated that most of the gonadal tissue is contained in the conical appendage when referring to *H. corrugata*, *H. fulgens* and *H. sorenseni*. While the proportion of gonadal tissue in the conical appendage may vary between species, the effectiveness of the MGBI as a measure of gonad growth is not diminished (at least within species), providing the rate of growth is consistent across the entire conical appendage. In light of the small volume of gonad in the conical appendage of *H. rubra* and *H. laevigata*, we took a slightly different measure of the conical appendage compared to previous studies on gonad volume. The apex of the visceral spire, rather than the base of the conical appendage (see Ault, 1984), was used as the posterior measurement point. Hence, the EGV and MGBI values are greater than they would have been with the standard method.

The MGBI estimates for blacklips (5–68 mm^3g^{-1}) and greenlips (5–58 mm^3g^{-1}) obtained here were similar or slightly lower than values for other species (Tutschulte and Connell, 1981; Ault, 1985; Wood and Buxton, 1996; Capinpin et al., 1998). By contrast, our upper MGBI estimate for greenlips is greater than that reported by Lleonart (1992) since he used whole body weight, rather than shucked weight in the estimates. While we found small differences in maximum MGBI between *H. rubra* and *H. laevigata*, Tutschulte and Connell (1981) reported much larger differences for Californian abalones, the maximum value for *H. corrugata* being 110 mm^3g^{-1} as opposed to 180 mm^3g^{-1} for *H. sorenseni*. Clearly, the volume occupied by the gonad, relative to the shucked weight of the animal differs between species. This in turn leads to species differences in both overall and weight-specific fecundity, bearing in mind that oocyte size varies between species (Sawatpeera et al., 2001).

The degree to which the EGV correlates with potential fecundity (i.e. egg counts from ovarian tissue) was not examined here as we believe that this measure of fecundity tends to overestimate the number of eggs that are actually spawned. The extent of the overestimate is dependent on the degree of partial spawning, absorption or necrosis of oocytes and/or the presence of multiple cohorts of oocytes in some species (e.g. Newman, 1967; Jebreen et al., 2000).

Given that proportion of gonad that resides in the conical appendage varies between species, meaningful inter-specific comparisons of MGBI values are not possible. However, providing that the rate of gonad growth is consistent across the conical appendage for a given species, this index does allow intra-specific comparisons of animals exposed to different conditions.

2.5.2 Oocyte development

The standardized diameter, area and volume of oocytes of *H. rubra* and *H. laevigata* increased proportionally with temperature and conditioning interval. The standardized diameters of oocytes recorded here were within the range of values previously reported for these species (Harrison and Grant, 1971; Shepherd and Laws, 1974; McShane et al., 1986; Lleonart, 1992), while oocyte areas were similar to those of *H. iris* and *H. australis* (Wilson and Schiel, 1995; Kabir, 2001).

Only Hahn (1994) has previously recorded oocyte volume for abalone. He calculated the maximum oocyte volume at 9.5×10^4 μm^3 for *H. discus hannai* using the formula for the volume of a sphere, which equates to a standardized diameter of 122 µm. This was less than the maximum mean oocyte diameters for *H. rubra* and *H. laevigata* reported here (143 µm and 160 µm, respectively). We calculated oocyte volume based on an ellipsoid shape and the data show that volume increases linearly during conditioning, whereas Hahn's (1994) work, using spherical volume, suggests an exponential increase. Hahn (1994) argued that conventional methods used to express oocyte size, such as oocyte diameter, resulted in over-emphasis of smaller oocytes and under-emphasis of larger

oocytes. He used the lesser diameter of stalked oocytes and the mean diameter of polygonal or round oocytes when calculating their volume. However, small, stalked oocytes are teardrop (almost elliptical) in shape, so using the lesser diameter of these oocytes under-estimated volume. Furthermore, using the mean diameter of the larger polygonal oocytes when calculating their volume leads to over-estimates as they are not perfectly round. By using the formula for the volume of an ellipsoid, we minimized both sources of error.

Comparing patterns in standardized residuals of blacklip and greenlip oocytes during conditioning with similar data from other haliotid studies is complicated by a number of factors. Both Lleonart (1992) and Moss (1998) expressed oocyte size as diameter, Kabir (2001) presented oocyte area, while Hahn (1994) calculated oocyte volume, using a different methodology. Furthermore, the range of oocyte size classes and sampling frequencies varied between the studies. If sampling is infrequent or oocyte size ranges too broad, this reduces the degrees of freedom for significance testing and the number of standardized residuals in the table. Hence, the difference between some residuals may not be significant, masking fine scale changes in oocyte development. We recommend that oocyte volume size classes be presented in geometric progression, as the rate of change in volume is much greater in large oocytes than in small ones.

The means of expressing conditioning interval is also important in presenting data on the dynamics of gonad or oocyte size of haliotids. Since each species has a different BZP, the EAT interval, rather than conditioning time (in days), should be used. Hahn (1994) identified this issue and related the EAT interval to oocyte volume and to tables of standardized residuals. However, Kabir (2001) presented conditioning times in days for *H. iris* and *H. australis*, but since he also determined the BZP's, the EAT conditioning intervals can also be calculated. Clearly, sampling frequency, the number of oocyte size categories and the means by which conditioning interval and oocyte size are expressed need to be sufficient to allow comparisons between such studies in future.

2.5.3 Conclusions

This study showed that the rate of gonad development in both blacklip and greenlip abalone was dependent on the cumulative difference between the holding temperature and the BZP. The BZP for gonadal development of blacklip abalone was 7.8ºC and that of greenlip abalone was 6.9ºC. The ODR during early oogenesis of both species was highly variable, but above a minimum diameter of 90µm oocyte shape was less variable and ODR approached 1. The use of the formula for the volume of an ellipsoid rather than of a sphere provided the more accurate estimate of actual oocyte volume. We recommend that future works on contingency table analysis of oocyte size frequency in abalone use ellipsoid volume and pay particular attention to sampling frequency, categorization of oocyte size classes and the means of expressing conditioning time (using EAT degree days).

2.6 Acknowledgements

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Chapter 3 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.

3.1 Abstract

Wild-caught blacklip (*Haliotis rubra*, Leach 1814) and greenlip (*H. laevigata*, Donovan 1808) abalone fed a formulated feed were held at 16ºC or 18ºC for different conditioning intervals ranging from 114-235 days and induced to spawn using ultraviolet-irradiated seawater. They were conditioned again for a second identical period before another induction. For *H. rubra*, mean spawning rate of both sexes was higher in groups held at 18ºC than at 16ºC, as was the repeat spawning rate. Conversely, animals held at 16ºC produced significantly more gametes than those at 18ºC. Egg production peaked (at 2.11 x 10^6 eggs female $^{-1}$) in groups held at 16ºC for ≥165 days (≥ 1350 EATºC-days). While both mean and total sperm production of *H. rubra* varied significantly, both figures were always high (1.0–9.0 x 10^{11} and 1.0–10 x 10^{12}, respectively). Unlike *H. rubra*, the spawning rate, repeat spawning rate and gamete production of both sexes of *H. laevigata* was higher when cultured at 16ºC than at 18ºC. Egg production peaked (at 1.93 x 10^6 eggs female $^{-1}$) in groups conditioned at 16ºC for ≥ 212 days (≥ 1930 EATºC-days). Both mean and total sperm production by *H. laevigata* was typically and order of magnitude lower (for a given body size) than for *H. rubra*. This study demonstrates that year-round hatchery production of seedstock of both species is possible providing broodstock are held under favorable environmental conditions, preferably 16ºC.

3.2 Introduction

Blacklip (*Haliotis rubra*) and greenlip (*H. laevigata*) abalone form the basis of a lucrative wild fishery and a rapidly expanding aquaculture industry in southern Australia. Culture of these species has generally relied upon the capture and induced spawning of wild broodstock but this process is often compromised by spatial and temporal variations in the availability of gravid broodstock and/or the stresses of capture and transport. Hence, a reliable means of ensuring continuity of larval supply is vital for consistent production of seedstock for farming.

Abalone broodstock can be conditioned and spawned predictably in the hatchery through the provision of a favourable physico-chemical environment. This includes a stable temperature that optimises gonad growth, high levels of dissolved oxygen, low levels of nitrites and ammonia and a pH of 7.5–8.5 (Fleming 2000). Broodstock must also be given a high quality feed in amounts slightly in excess of their needs, as poor feeds or low feeding rations compromise gamete maturation and spawning rates (Uki & Kikuchi 1982a).

When nutrition is not limiting, temperature is the main factor influencing the rate of gonad development in most species of abalone. Its effect is cumulative above a certain threshold temperature that varies between species. Kikuchi & Uki (1974a,b) were the first to record this phenomenon, and named the threshold temperature the “biological zero point” (BZP). By subtracting the BZP from the daily water temperature and summing this figure over the culture time (in days) they were able to describe the Effective Accumulative Temperature in degree days (EATºC-days) for gonad conditioning of two Japanese abalone species. At present, there is only one account of the EAT for conditioning of southern hemisphere abalones, that of Kabir (2001) on *H. australis* and *H. iris*.

Previous reports on broodstock conditioning of blacklip and greenlip abalone have generally concentrated on a single species held at one temperature. Lleonart (1992) described changes in gonad histology and volume during conditioning of

greenlip abalone held at 16ºC but did not examine spawning success. Plant (2002) conditioned *H. laevigata* at 17ºC and observed spawning response relative to a range of temperature treatments applied 1-4 days prior to induction. Those groups exposed to an increase of 5ºC over this interval showed the best results. Savva, Heasman & Brand (2000) examined the effect of broodstock diet on fecundity and egg quality in *H. rubra* conditioned at 16ºC. Broodstock on a formulated feed appeared to spawn more eggs and produce better quality larvae than those fed the brown alga *Phyllospora comosa* or a mix of both diets. Plant, Mozquiera, Day & Huchette (2002) documented the spawning success of groups of blacklip abalone held at 18ºC for periods of 30–150 days. The spawning response of each sex was highest in the group cultured for 120 days. Surprisingly, the proportion of female spawners was always higher than that of males, whilst the reverse is usually the case for this species (*pers. obs.*).

The aim of this study was to determine the optimal conditioning regime for repeat spawnings of wild-caught blacklip and greenlip abalone broodstock conditioned on a formulated feed. This was achieved by holding both sexes of each species at temperatures of either 16ºC or 18ºC for one of five different conditioning intervals (calculated as EATºC-days) over two cycles. The optimal regime was defined as the combination of time and temperature that maximised spawning response, in terms of the proportion of spawners (and repeat spawners) and gamete production.

3.3 Materials and methods

3.3.1 Broodstock collection

Greenlip abalone broodstock (100–120 mm shell length) were collected on 26 November 2001 near Flinders Island (between Vansittart and Puncheon Islands) and held in flow-through tanks at ambient temperature for 3 days. During this time it was noticed that some animals spawned. Information from the fisher indicated that spawning commenced in the wild during the previous 4 weeks. Blacklip abalone broodstock (100–130 mm shell length) were collected on 3

December 2001 near Swan Island from FRV Challenger and some animals spawned in the boat's holding tanks. Both species are known to be sexually mature at these sizes and locations (Tarbath & Officer 2003, C. Mason, pers. comm.).

Animals were transferred to the Tasmanian Aquaculture and Fisheries Institute, Marine Research Laboratories and held in 600 L tanks supplied with ambient temperature seawater until examination and allocation to experimental treatments. They were measured, weighed, numerically tagged and gonad condition assessed by visual score according to the following criteria: 0 = sex indistinguishable; 1 = sex distinguishable, thin gonad with pointed tip; 2 = gonad partially enlarged with pointed tip; 3 = gonad swollen with rounded tip. All animals were scored as 0 or 1 and allocated a putative spawning date of 15 November 2001.

3.3.2 Experimental design

At the start of the experiment, 10 to 15 abalone of each sex and species were randomly assigned either 16ºC or 18ºC (the minimum and maximum temperatures typically used to condition these species; Fleming, 2000) and five conditioning intervals (1200, 1400, 1600, 1800 or 2000 EATºC-days, referred to in the text as 1200 group, 1400 group etc.). Animals of each sex and species were held separately, with 3 tanks for each temperature treatment (i.e. 2 x 2 x 3 x 2 = 24 tanks in total with ~25 animals $tank^{-1}$). The EAT was calculated using an estimated biological zero point (BZP) of 7.5ºC for both species (based on the range of values for other species given in Kikuchi & Uki 1974a,b). The BZP was subtracted from the actual holding temperature (e.g. 16-7.5 = 8.5ºC or 18-7.5 = 10.5ºC) and summed over the number of days that animals were held before spawning. At 16ºC, EAT intervals of 1200, 1400, 1600, 1800 and 2000 equated to 141, 165, 188, 212 and 235 days, respectively. At 18ºC, they corresponded to 114, 133, 152, 171 and 190 days, respectively. The true BZP values were calculated at the end of a concurrent study that examined gonad development at temperatures from 12ºC–18ºC (Grubert & Ritar 2004).

3.3.3 Husbandry and monitoring

Broodstock were conditioned in 150 L round fibreglass tanks receiving flow-through seawater at rate of 1.5 L min^{-1} $tank^{-1}$. Animals were fed daily to satiation on a broodstock conditioning feed (Adam and Amos Abalone Foods Pty Ltd, Mount Barker, SA, Australia). Mortalities and spontaneous spawnings were recorded during feeding. Wastes were siphoned from each tank every second day. Photoperiod was maintained at 12L:12D starting at 06:00h and light intensity at the bottom of the tanks (when filled) was 90–100 Lux. Water temperature was recorded using StowAway TidbiT temperature loggers (Onset Computer Corporation, Bourne, MA, USA) with the means and standard errors of the two experimental temperatures being 16.2 ± 0.2ºC and 18.1 ± 0.3ºC, respectively.

3.3.4 Induction of spawning

On each induction day, all animals from the group scheduled for induction were removed from their holding tanks at 09:00h. The VGI of each animal was recorded prior to being placed into a 14 L polyethylene spawning tray containing 10 L seawater. Water temperature in the trays was the same as the conditioning temperature (i.e. 16ºC or 18ºC). The trays were then covered in black plastic panels to exclude light. The induction procedure involved the simultaneous application of ultraviolet (UV) irradiated seawater and temperature change. One micron filtered seawater was irradiated using 2 x 150 W UV sterilization units (Wedeco Pty Ltd, Girraween, NSW, Australia), the quartz sleeves of which were cleaned after every 50 hours of use. Water temperature was raised by 1ºC h^{-1} for 4 h then allowed to return to the original temperature at the same rate (i.e. 16ºC→20ºC→16ºC or 18ºC→22ºC→18ºC). Flow rate to each tray was 300 ml per minute. Animals were monitored from 14:00–22:00h and the time that they began spawning recorded. Once an animal began spawning, the supply of UV irradiated seawater was stopped facilitating the retention of gametes.

When males spawned prior to 22:00h, sperm water was poured off, made up to a known volume, mixed and samples taken for density estimates (using absorbance at 340 nm; Ritar & Grubert 2002). The male trays were then refilled with fresh seawater. Both sexes were held overnight in their spawning trays with no water exchange. Each tray was aerated and a stable temperature maintained using a waterproof, thermostatically controlled heat mat (Thermofilm Australia Pty Ltd, Victoria). The following morning, samples of sperm water (for sperm density estimates) were taken from those males that had continued, or started, spawning after 22:00h. At the same time, eggs released from each female were suspended in 10 L of seawater and subsampled (1 ml) in triplicate to estimate fecundity. When sperm were collected from the same male on two occasions (at night and the following morning), sperm production estimates were summed. Once all gametes were collected, the abalone were returned to their conditioning tanks.

2.3.5 Statistics

For both sexes of each species, analyses of gamete production and spawning response time data were conducted using one-way and two-way (without replication) analysis of variance (ANOVA). Tukey-Kramer HSD tests were used for post-hoc comparison. Bartlett's tests were used to check for homogeneity of variance and where necessary (i.e. p-value for the F-ratio < 0.05) the data were √transformed (Sokal & Rohlf 1995) and re-analyzed. Probabilities of < 0.05 (for the ANOVA's) were considered significantly different. Significant interactions between temperature and EAT conditioning interval are denoted by T*EAT; non-significant differences are expressed as n.s; data are presented as mean ± SEM. The ANOVA tests on gamete production by each sex and species were structured in a number of ways. One analysis examined the effect of temperature and conditioning interval on mean gamete production of each EAT group at the first induction (i_1) then at the second induction (i_2), using data from only those animals that had spawned. A second analysis examined how total gamete production at both inductions (i.e. $x_i = i_1 + i_2$, where x_i = each individual that spawned one or more times) varied relative to temperature and EAT conditioning interval. These

values are referred to as IndTotal in the text. Due to lack of independence in data from successive inductions (as gamete production at the first induction may affect that at the second), no direct comparisons of the first and second inductions were made (i.e. it was not appropriate to use two-way ANOVA with replication). However, the difference in gamete production between the first and second inductions was calculated for each individual that spawned at least once ($x_i = i_2 - i_1$) and standardized by adding the absolute value of the largest negative value (x_{min}) plus one (i.e. $x_i = x_i + |x_{min}| + 1$). These data are referred to as IndDiff in the text. One-way ANOVA was employed to test for differences in mean spawning, repeat spawning and mortality rates between each temperature treatment (percentage data was arcsine √transformed prior to analysis). G-tests were used to check for independence between the frequencies of animals that spawned zero, one or two times and EAT conditioning interval. If the frequencies of each spawning group were independent of EAT for 16ºC and 18ºC groups then these values were pooled over all EAT categories and another G-test used to examine temperature effects on spawning patterns. Regression analyses were used to test relationships between shell length and gamete production. Statistics were executed using Excel 2000 (Microsoft, Redmond, WA, USA) and JMP version 5.0 (SAS Institute, Cary, NC, USA).

3.4 Results

3.4.1 Spawning response of female blacklip abalone (H. rubra)

At the first induction, the mean spawning rate of female blacklip groups held at 16ºC and 18ºC was 49 ± 4% and 63 ± 3%, respectively (n.s., Table 3.1). Mean spawning response times were 6h 32 ± 17 min at 16ºC and 6h 05 ± 13 min at 18ºC (n.s.). Overall mean egg production for spawning females was not significantly different between temperature treatments (1.39 ± 0.65 and 0.71 ± 0.18 x 10^6 eggs $female^{-1}$ at 16ºC and 18ºC, respectively). However, one group (1600) at 18ºC produced significantly more eggs than the other groups held at this temperature.

At the second induction, spawning rate in the 18ºC treatment was significantly higher than the 16ºC treatment (80 ± 9% and 43 ± 2%, respectively), as was the

number of repeat spawners (i.e. animals spawning at both inductions; 46 ± 9% and 32 ± 2%, respectively). The proportion of females that spawned either repeatedly, only once, or not at all did not differ significantly between conditioning intervals when females were induced a second time. Hence, it was possible to compare the spawning patterns of all animals at both temperatures. The number of repeat spawners was significantly higher at 18ºC than at 16ºC, while the number of non-spawners was highest in the 16ºC group. Mean response times were not significantly different; 7h 02 ± 20min at 16ºC and 6h 25 ± 10 min at 18ºC. Mortalities were 5% at 16ºC and 16% at 18ºC.

There was a significant T*EAT interaction on the group mean egg production of animals at the second induction. Of the females held at 16ºC, those in the 1600 group produced significantly fewer eggs (0.20 x 10^6 female^{-1}) than the other groups (0.80–2.11 x 10^6 female^{-1}). In the case of the animals held at 18ºC, the 1200 group produced significantly more eggs (1.59 x 10^6 female^{-1}) than all other groups (0.20–0.65 x 10^6 female^{-1}).

There were no significant temperature or conditioning interval effects on the difference in egg production between the first and second inductions (IndDiff), whereas total gamete production across both inductions (IndTotal) was significantly higher for groups at 16ºC than at 18ºC. There was no significant relationship between shell length and egg production (r^2 = 0.001).

3.4.2 Spawning response of male blacklip abalone (H. rubra)

At the first induction, spawning rate of male blacklip abalone in both temperature treatments was high (93 ± 5% and 97 ± 3% at 16ºC and 18ºC, respectively; n.s., Table 1). Mean spawning response times were 6h 15 ± 19min at 16ºC and 5h 45 ± 8min at 18ºC. While these means were not significantly different, there was a T*EAT interaction in spawning response times, but there was no obvious trend in the data. Mean sperm production was significantly higher in the 16ºC treatment (4.12 ± 0.84 x 10^{11} male^{-1}) than the 18ºC treatment (2.75 ± 0.75 x 10^{11} male^{-1}) and in both cases the 1800 group produced the greatest mean sperm number (8.88 ± 1.31 x 10^{11} and 4.77 ± 1.17 x 10^{11} male^{-1} at 16ºC and 18ºC, respectively).

Table 3.1 Spawning rate, gamete production (x 10^6 for females and x 10^{11} for males) and repeat spawning rate at successive inductions of blacklip abalone relative to sex, temperature (T°C) and conditioning interval (EAT). n = sample size, Mort = mortalities between inductions. Comparisons made within sex and within column. EAT groups (at each temperature) with the same lower case letter are not significantly different. Likewise, means for each temperature treatment with the same upper case letter are not significantly different. T*EAT superscript indicates an interaction effect (see text for details of each case).

				Induction 1			Induction 2		
Sex	T°C	EAT	n	Percentage spawning	Gamete production mean (± s.e.)	Mort	Percentage spawning	Gamete production mean (± s.e.)	% repeat spawners
Female	16	1200	12	17	1.98 ± 1.84^{a}	2	50	0.80 ± 0.20^{a}	20
		1400	12	58	1.05 ± 0.29^{a}	1	46	2.11 ± 0.69^{a}	36
		1600	12	67	1.26 ± 0.41^{a}	0	42	0.20 ± 0.06^{b}	33
		1800	11	64	0.92 ± 0.28^{a}	0	36	0.77 ± 0.29^{a}	27
		2000	12	50	1.76 ± 0.45^{a}	0	42	1.70 ± 0.46^{a}	42
		mean ± s.e.		49 ± 4^{A}	1.39 ± 0.65^{A}		43 ± 2^{B}	$1.12 \pm 0.34^{T*EAT}$	32 ± 2^{B}
	18	1200	15	67	0.67 ± 0.09^{b}	5	70	1.59 ± 0.58^{a}	40
		1400	13	46	0.82 ± 0.20^{b}	3	70	0.31 ± 0.14^{b}	30
		1600	10	60	1.49 ± 0.39^{a}	0	100	0.20 ± 0.05^{b}	60
		1800	12	83	0.46 ± 0.19^{b}	2	100	0.65 ± 0.25^{b}	80
		2000	12	58	0.10 ± 0.05^{b}	0	58	0.60 ± 0.29^{b}	42
		mean ± s.e.		63 ± 3^{A}	0.71 ± 0.18^{A}		80 ± 9^{A}	$0.67 \pm 0.26^{T*EAT}$	46 ± 9^{A}
Male	16	1200	12	100	1.13 ± 0.37^{c}	0	92	1.46 ± 0.41^{b}	90
		1400	12	75	1.83 ± 0.57^{bc}	0	75	3.48 ± 0.73^{ab}	50
		1600	13	100	4.65 ± 0.77^{ab}	0	92	5.25 ± 0.69^{a}	92
		1800	12	100	8.88 ± 1.31^{a}	2	90	3.70 ± 1.00^{ab}	90
		2000	13	92	4.58 ± 1.19^{ab}	1	67	3.99 ± 1.08^{ab}	58
		mean ± s.e.		93 ± 5^{A}	4.21 ± 0.84^{A}		83 ± 5^{A}	$3.58 \pm 0.78^{T*EAT}$	76 ± 9^{B}
	18	1200	11	100	1.21 ± 0.30^{a}	1	100	1.42 ± 0.33^{b}	100
		1400	12	100	1.69 ± 0.52^{a}	0	100	0.99 ± 0.42^{b}	100
		1600	12	100	2.35 ± 0.50^{a}	0	100	0.93 ± 0.23^{b}	100
		1800	11	100	4.77 ± 1.17^{a}	1	80	1.21 ± 0.94^{b}	90
		2000	12	83	3.73 ± 1.26^{a}	0	92	5.93 ± 1.04^{a}	83
		mean ± s.e.		97 ± 3^{A}	2.75 ± 0.75^{B}		94 ± 4^{A}	$2.10 \pm 0.59^{T*EAT}$	93 ± 3^{A}

Spawning rate at the second induction was also high in both temperature treatments (83 ± 5% at 16ºC and 94 ± 4% at 18ºC, n.s.). The proportion of repeat spawners was 76 ± 9% and 93 ± 3% at 16ºC and 18ºC, respectively. Since the proportion of males that spawned either repeatedly, only once, or not at all did not differ significantly between conditioning intervals at each temperature, this allowed a comparison of spawning patterns between animals held at 16ºC and 18ºC. Most males spawned twice, with the proportion of repeat spawners highest in the 18ºC groups and the proportion of animals that spawned only once highest in the 16ºC group. Spawning commenced after 6h 41 ± 18min and 6h 24 ± 11min at 16ºC and 18ºC, respectively. Again, there was a significant interaction between

temperature and conditioning interval on spawning response times but no obvious trend in the data was discernible. Mortalities were low at both temperatures; 5% at 16ºC and 3% at 18ºC.

There was a significant T*EAT interaction on mean sperm production of animals at the second induction. At 16ºC, males in the 1600 and 1200 groups had the highest ($5.25 \pm 0.69 \times 10^{11}$ male^{-1}) and lowest ($1.46 \pm 0.41 \times 10^{11}$ male^{-1}) sperm production respectively, whereas at 18ºC, males in the 2000 group produced significantly more sperm ($5.93 \pm 1.04 \times 10^{11}$ male^{-1}) than all other groups ($0.93–1.42 \times 10^{11}$ male^{-1}).

There was a significant interaction between temperature and conditioning interval on the means of the standardized difference in sperm production between the first and second inductions (IndDiff). However, the interaction was influenced by the 2000 group at 18ºC which showed a significantly greater increase in sperm production between inductions than all others. A T*EAT interaction was also detected for total sperm production over both inductions (IndTotal). IndTotal increased proportionally with EAT, although this difference was largely driven by animals in the 16ºC groups, which typically produced more sperm than the 18ºC groups. There was no significant relationship between shell length and sperm production ($r^2 = 0.001$).

3.4.3 Spawning response of female greenlip abalone (H. laevigata)

At the first induction, the mean spawning rate of female greenlip groups was 56 ± 11% at 16ºC and 37 ± 5% at 18ºC (n.s., Table 2). Mean spawning response times were not significantly different between temperatures treatments (9h 06 ± 18min at 16ºC and 9h 15 ± 36min at 18ºC) and there was no EAT effect on this variable. Significant temperature and EAT conditioning interval effects on mean egg production were detected. Females held at 16ºC produced more eggs ($1.17 \pm 0.32 \times 10^6$ female^{-1}) than those held at 18ºC ($0.38 \pm 0.18 \times 10^6$ female^{-1}) and the 1400 group at 16ºC produced fewer eggs (0.36×10^6 female^{-1}) than the remaining groups at that temperature ($0.93–1.69 \times 10^6$ female^{-1}).

When induced a second time, the mean spawning rate of groups at 16ºC was 68 ± 14% and at 18ºC was 64 ± 13% (n.s.). Mean repeat spawning rate was 47 ± 2% and 28 ± 9% at 16ºC and 18ºC, respectively. For females held at 16ºC, there was a significant EAT effect on the proportion of females that spawned either repeatedly, only once, or not at all. The 1200 group contained significantly more non-spawners and fewer repeat spawners, than the other groups. There was no EAT effect on the frequency of non-, single or repeat spawners in the 18ºC treatment. Spawning commenced after 9h 12 ± 23min at 16ºC and 8h 48 ± 37min at 18ºC and there was no temperature or EAT effect on this variable. Mortality rates were 5% and 9% at 16ºC and 18ºC, respectively (n.s.). Females held at 16ºC produced significantly more eggs ($1.59 \pm 0.54 \times 10^6$ female^{-1}) than those held at 18ºC ($0.99 \pm 0.24 \times 10^6$ female^{-1}) but conditioning interval did not affect egg production at either temperature.

There were no significant temperature or EAT effects on the mean of IndDiff across the range of treatments, implying no change in egg production between the first and second inductions. There was however, a significant temperature effect on IndTotal in which egg totals for groups at 16ºC were higher than at 18ºC. There was no significant relationship between shell length and egg production ($r^2 = 0.003$).

3.4.4 Spawning response of male greenlip abalone (H. laevigata)

At the first induction, mean spawning rate of greenlip males at 16ºC (77 ± 9%) was significantly higher than that of males at 18ºC (42 ± 10%; Table 2). Spawning commenced after 9h 17 ± 31min at 16ºC and 8h 48 ± 26min at 18ºC, with no temperature or EAT effects on this factor. A significant T*EAT interaction on mean sperm production was detected, being greater for animals held at ≥1600 EATºC-days at 16ºC ($5.81–12.38 \times 10^{10}$ male^{-1}) than remaining groups at 16ºC ($0.06–0.07 \times 10^{10}$ male^{-1}) and all groups at 18ºC ($0.08–0.67 \times 10^{10}$ male^{-1}).

Table 3.2 Spawning rate, gamete production (x 10^6 for females and x 10^{10} for males) and repeat spawning rate at successive inductions of greenlip abalone relative to sex, temperature (T°C) and conditioning interval (EAT). n = sample size, Mort = mortalities between inductions. Comparisons made within sex and within column. EAT groups (at each temperature) with the same lower case letter are not significantly different. Likewise, means for each temperature treatment with the same upper case letter are not significantly different. T*EAT superscript indicates an interaction effect (see text for details of each case).

				Induction 1			Induction 2		
Sex	T°C	EAT	n	Percentage spawning	Gamete production mean (± s.e.)	Mort	Percentage spawning	Gamete production mean (± s.e.)	% repeat spawners
Female	16	1200	13	31	1.20 ± 0.47^{a}	1	17	1.57 ± 1.02^{a}	8
		1400	11	83	0.36 ± 0.08^{b}	0	73	1.53 ± 0.29^{a}	63
		1600	12	33	1.69 ± 0.40^{a}	2	100	1.27 ± 0.32^{a}	30
		1800	10	60	0.93 ± 0.25^{a}	0	75	1.93 ± 0.62^{a}	60
		2000	12	75	1.69 ± 0.42^{a}	0	75	1.66 ± 0.47^{a}	75
		mean ± s.e.		56 ± 11^{A}	1.17 ± 0.32^{A}		68 ± 14^{A}	1.59 ± 0.54^{A}	47 ± 2^{A}
	18	1200	12	25	0.71 ± 0.50^{a}	1	36	0.69 ± 0.01^{a}	9
		1400	12	50	0.33 ± 0.07^{a}	0	33	0.98 ± 0.42^{a}	25
		1600	12	25	0.10 ± 0.04^{a}	2	70	0.95 ± 0.33^{a}	10
		1800	11	45	0.25 ± 0.07^{a}	0	100	0.75 ± 0.22^{a}	45
		2000	12	42	0.53 ± 0.21^{a}	2	80	1.56 ± 0.24^{a}	50
		mean ± s.e.		37 ± 5^{A}	0.38 ± 0.18^{B}		64 ± 13^{A}	0.99 ± 0.24^{B}	28 ± 9^{B}
Male	16	1200	12	55	0.07 ± 0.02^{cd}	3	89	6.03 ± 3.08^{a}	33
		1400	11	91	0.06 ± 0.02^{d}	1	80	13.72 ± 5.85^{a}	70
		1600	11	100	12.38 ± 4.86^{a}	2	78	26.80 ± 9.50^{a}	66
		1800	12	58	5.81 ± 2.01^{abc}	2	80	20.21 ± 5.57^{a}	55
		2000	12	83	7.99 ± 2.33^{ab}	0	92	25.86 ± 5.03^{a}	75
		mean ± s.e.		77 ± 9^{A}	$5.26 \pm 1.85^{T*EAT}$		84 ± 3^{A}	18.50 ± 5.81^{A}	60 ± 7^{A}
	18	1200	12	25	0.67 ± 0.17^{a}	2	80	5.85 ± 1.49^{a}	20
		1400	12	25	0.12 ± 0.04^{a}	1	100	5.07 ± 1.95^{a}	18
		1600	12	75	0.08 ± 0.02^{a}	0	100	7.06 ± 2.74^{a}	75
		1800	11	27	0.27 ± 0.11^{a}	2	78	4.00 ± 2.08^{a}	11
		2000	12	58	0.80 ± 0.43^{a}	2	80	4.21 ± 2.25^{a}	40
		mean ± s.e.		42 ± 10^{B}	$0.39 \pm 0.15^{T*EAT}$		88 ± 5^{A}	5.24 ± 2.10^{B}	31 ± 12^{B}

When induced a second time, the spawning rate of all male groups was high; 84 ± 3% at 16ºC and 88 ± 5% at 18ºC. The mean repeat spawning rate was 60 ± 7% and 31 ± 12% at 16ºC and 18ºC, respectively (n.s.). At 16ºC, the proportion of males that spawned either repeatedly, only once, or not at all did not differ significantly between conditioning intervals. However, at 18ºC, most males in the 1200, 1400 and 1800 groups spawned only once, whereas most males in the 1600 group spawned twice. In the 2000 group, the ratio of repeat spawners to single spawners was 2:3. The average spawning response time was 8h 09 ± 26min at 16ºC and 8h 50 ± 14min at 18ºC. There was a significant T*EAT interaction on spawning response times but no obvious trend in the data was discernible. Mortality rates were 16% at 16ºC and 13% at 18ºC. Males held at 16ºC produced

significantly more sperm ($18.50 \pm 5.81 \times 10^{10}$ male^{-1}) than those held at 18ºC ($5.24 \pm 2.10 \times 10^{10}$ male^{-1}) but conditioning interval did not affect sperm production at either temperature.

Values of IndDiff (the standardized difference in sperm production between the first and second inductions) for animals held at 16ºC were significantly higher than those at 18ºC, indicating that the increase in sperm production between the two inductions was greatest for 16ºC males. There was a significant T*EAT interaction on the means of the individual totals over both inductions (IndTotal). IndTotal increased proportionally with EAT in the 16ºC groups, with a three-fold difference between the longest and shortest conditioning intervals. No significant difference in this factor was detected at 18ºC. There was no significant relationship between shell length and sperm production ($r^2 = 0.032$).

3.5 Discussion

3.5.1 Spawning rate and gamete production

Conditioning of blacklip and greenlip abalone on a formulated feed for as little as 114 days at 18ºC (1200 EATºC-days) or as long as 235 days at 16ºC (2000 EATºC-days) before induction generally yielded gamete production and spawning rates that can be considered commercially acceptable for hatchery production. Likewise, inductions after a second, identical conditioning interval also resulted in moderate to high spawning success in most cases.

Although this study used an estimated BZP of 7.5ºC for both species, a concurrent work showed that the actual BZP for gonad development was 7.8ºC for blacklips and 6.9ºC for greenlips (Grubert & Ritar, 2004). Therefore, at any given temperature, it takes less time for greenlips, and longer for blacklips, to reach the designated conditioning interval than when the estimated BZP value was used in the original calculations. The recalculated EAT values (using the actual BZP) for each species are given in Table 3.3.

Table 3.3 Estimated EAT, based on a BZP of 7.5ºC, and true EAT for blacklip and greenlip abalone, based on BZP values of 7.8ºC and 6.9ºC, respectively (Grubert & Ritar, 2004). True EAT is calculated using a water temperature of 16ºC.

Estimated	True EAT	
EAT	Blacklip	Greenlip
1200	1160	1280
1400	1350	1500
1600	1540	1700
1800	1740	1930
2000	1930	2140

The mean (and total) gamete production of both sexes of each species was higher when broodstock were held at 16ºC than at 18ºC. The optimal conditioning interval to ensure a high spawning rate and gamete production for blacklip abalone at 16ºC is ≥188 days (≥1540 EATºC-days) for males and ≥165 days (≥1350 EATºC-days) for females. Corresponding figures for *H. laevigata* are ≥188 days (≥1700 EATºC-days) for males and ≥212 days (≥1930 EATºC-days) for females.

The fact that reproductive conditioning of adult *H. rubra* and *H. laevigata* was more successful at 16ºC than at 18ºC was unexpected, particularly given that the optimal temperature for growth of juveniles is 17.0ºC and 18.3ºC, respectively (Gilroy & Edwards 1998). Thus, it appears that the optimal temperature for growth (be it somatic or gonadal) of *H. rubra* and *H. laevigata* declines with size, a phenomenon previously documented in *H. rufescens* by Steinarsson and Imsland (2003).

Given that fecundity of abalone can be determined in a number of ways, it is important to distinguish between estimates of instantaneous fecundity (derived from counts of spawned eggs), as in this study, and potential fecundity (number of oocytes in the ovary). This distinction is made as latter tends to over-estimate the former, particularly when the ovary contains multiple cohorts of oocytes (e.g. in *H. asinina*; Jebreen, Counihan, Fielder & Degnan 2000) or in the case of partial

spawning. As we subsampled spawned eggs, the following discussion refers only to estimates of instantaneous fecundity.

The spawning response and egg production of *H. rubra* reported here was similar or slightly higher (for equivalent-sized animals) to previous research on this species (see Table 3.4). Plant et al. (2002) found that these parameters in female *H. rubra* peaked after 120 days conditioning at 18ºC (92% spawned; 2.0 x 10^6 eggs female^{-1}), which equates to approximately 1220 EATºC-days, while females conditioned for longer (150 days or approximately 1600 EATºC-days) were less responsive (65% spawned) and produced fewer eggs (1.1 x 10^6 eggs female^{-1}). By contrast, no reduction in egg production by female *H. rubra* held for ≥1200 EATºC-days was observed during this study.

Savva et al. (2000) found that for *H. rubra* held at 16ºC and repeatedly induced at intervals of 12 weeks, an average of only 8% of females spawned at each induction, producing 1.6 x 10^6 eggs female^{-1} (Table 3.4). Only 4% of females spawned more than once, suggesting that a conditioning cycle of 12 weeks (approximating 690 EATºC-days) was insufficient to complete oogenesis. However, these animals were collected from the northern limit of the species range where surface water temperatures can reach 25ºC in summer. Since high temperatures are stressful to abalone (Gilroy & Edwards 1998), the reproductive performance of the broodstock used by Savva et al. (2000) may have been compromised by their thermal history.

Maximum egg production estimates for *H. laevigata* (of a given size) reported here are greater than most estimates in previous works, with the exception of Babcock and Keesing (1999). These authors used larger animals (see Table 3.4) and recorded a maximum fecundity of 8.2 x 10^6 eggs, approximately 2 million more than our highest estimate. As for *H. rubra*, there was no relationship between shell length and egg (or sperm) production in *H. laevigata* in this study.

Lleonart (1992) conditioned wild-caught *H. laevigata* for 112 days at 16ºC (approximately 1020 EATºC-days). Only 38% of females and 17% of males spawned when induced, resulting in the production of 0.1–1.0 x 10^6 eggs female^{-1}, while no sperm production was given. Interestingly, when smaller (60–80 mm SL), cultured *H. laevigata* to were induced, the size-specific fecundity was greater than that of larger (120–175 mm SL) wild-caught conditioned animals (2700–5775 eggs mm^{-1} SL and 833–5714 eggs mm^{-1} SL respectively; absolute values in Table 3.4).

When Plant (2002) induced wild-caught *H. laevigata* that were previously conditioned (but at different stages of maturity) at 17ºC, the initial spawning rate was only 10%–40% for females and 13%–50% for males. At the second induction 4 months (approximately 1030 EATºC-days) later, spawning rate was somewhat higher, 14%–60% for females and 13%–100% for males. This compares to spawning rates of 17%–100% for females and 25%–100% for males during this study. Egg production, which was recorded by Plant (2002) only at the second induction, ranged from 0.1–1.4 x 10^6 eggs female^{-1}, while there was a wider range (0.03–5.9 x 10^6 eggs female^{-1}) of estimates in our study.

Groups of wild-caught *H. laevigata* conditioned at 17ºC and induced repeatedly at intervals of 6–12 weeks (equivalent to 420–840 EATºC-days, respectively) produced 0.1–1.5 x 10^6 eggs female^{-1} (K. Freeman, pers. comm.) which is comparable to the values derived from our data (0.1–1.3 x 10^6 eggs female^{-1}; calculated as total egg production/number of animals induced, not number of animals that spawned). However, animal weights in the former study were approximately 25% heavier than here (see Table 3.4 for comparison of SL). In that study, total egg production in the two shortest cycles (420 and 560 EATºC-days) was low at the first induction and high at the second (equivalent to 840 and 1120 EATºC-days, respectively), whereas the reverse was the case for longer cycles (700 and 840 EATºC-days). Beyond the second induction, total egg production progressively declined in all groups. This suggests that female *H. laevigata* can be conditioned within 700–1120 EATºC-days at 17ºC, which is

Table 3.4 Instantaneous fecundity (I.F.) from induced spawnings of selected female Haliotidae relative to shell length, origin and diet. + = mean of all animals induced; Dash = data not available; Cult. = Cultured broodstock; CWC = Conditioned wild-caught broodstock; WC = Wild-caught broodstock; G. b. = *Gracilariopsis bailinae*; * = Adam and Amos Abalone Feeds (Pty Ltd) broodstock feed; P. c. = *Phyllospora comosa*; N. l. = *Nereocystis luetkeana*; P. m. = *Palmaria mollis*.

Species	I.F. (x 10^3) mean ± s.d. or range	Shell Length (mm) mean ± s.d. or range	Origin	Diet	Source
H. asinina	102 ± 1	49 ± 1	Cult.	G. b.	Bautista-Teruel,
	137 ± 1			G. b. and formulated	Millamena & Fermin (2001)
	126 ± 2			formulated	
H. australis	0.2 – 900	75 ± 11	CWC	Various macroalgae	Moss (1998)
H. coccinea canariensis	11 – 75	28 – 48	CWC	–	Pena (1986)
H. iris	3 – 1 750	–	WC	–	Moss, Illingworth & Tong (1995)
	1 000 – 7 000	125 – 140	CWC	–	G. Moss, pers. comm.
H. laevigata	100 – 1 000	120 – 175	CWC	Various macroalgae	Lleonart (1992)
	162 – 508	60 – 88	Cult.	Various macroalgae	
	340 – 8 200	117 – 196	WC	–	Babcock & Keesing (1999)
	100 – 1 400	107 – 142	CWC	formulated*	Plant (2002)
	100 – 1 500$^+$	88 – 142	CWC	formulated*	K. Freeman, pers. comm.
	15 – 5 900	100 – 120	CWC	formulated*	This study
H. rubra	1 910 ± 290	100 – 125	CWC	formulated*	Savva et al. (2000)
	1 710 ± 570			P. c. and formulated*	
	1 110 ± 300			P. c.	
	54 – 5 900	120 – 142	WC	–	Litaay & De Silva 2001
	20 – 2 600	109 ± 1	CWC	formulated*	Plant et al. (2002)
	15 – 4 800	100 – 130	CWC	formulated*	This study
H. rufescens	112 – 5 300	111 – 194	WC	–	Ault (1985)
	85 – 11 085	65 – 182	CWC	N. l.	
	242 ± 263	92 ± 6	Cult.	N. l.	Buchal, Levin
	181 ± 133	89 ± 5	Cult.	P. m.	& Langdon (1998)
H. tuberculata	20 – 1 600	–	WC	–	Clavier (1992)

shorter than the optimal figure suggested in our study (>1930 EATºC-days at 16ºC). However, the reduction in egg production over a series of frequent inductions indicates that short conditioning periods (i.e. <1200 EATºC-days) are not conducive to consistent spawnings of this species.

Kabir (2001) found that the intervals required to condition the cool temperate abalone *H. australis* and *H. iris* to oocyte maturation (determined histologically) were moderate to long. *H. australis* (BZP = 5.0ºC) required ≥1400 EATºC-days, similar to that for female *H. rubra* and *H. laevigata* (this study) and *H. discus hannai* (Kikuchi & Uki 1974a), whereas *H. iris* (BZP = 6.2ºC) required ≥2700 EATºC-days, comparable to the figure for *H. discus* (Kikuchi & Uki 1974b).

The interval required for oogenesis of the tropical abalone, *H. asinina*, is short, ranging from 28–40 d during the spawning season (i.e. October to April; Jebreen, Counihan, Fielder & Degnan 2000). This equates to 310–440 EATºC-days when calculated using the BZP for larval development (i.e. 15ºC; Sawatpeera, Upatham, Kruatrachue, Chitramvong, Songchaeng, Pumthong & Nugranad 2001) as a proxy for gonad development and a temperature of 26ºC (the mean seawater temperature during the spawning season; Counihan, McNamara, Souter, Jebreen, Preston, Johnson & Degnan 2001). This interval range is less than one third that required for oogenesis of temperate abalone.

The physiological reasons behind the large inter-specific differences in the EAT conditioning interval required for gametogenesis of abalone may relate to species differences in the activity and/or complexity of the biochemical pathways that control this process. Further studies on the hormonal control of gametogenesis in haliotids relative to the EAT conditioning interval need to be undertaken to determine the factors responsible for these differences.

3.5.2 Fecundity and body size

Despite the large number of females induced to spawn during this study (> 100 for both species), we found no relationship between egg production and body size (as shell length) in either *H. rubra* or *H. laevigata*. Similar observations were reported by Babcock and Keesing (1999) for *H. laevigata* and Clavier (1992) for *H. tuberculata*, although we note that the latter author plotted instantaneous fecundity against body weight.

By contrast, Ault (1985) and Litaay and De Silva (2001) reported power and quadratic relationships between egg production and shell length in *H. rufescens* and *H. rubra*, respectively. However, we are unsure of the reliability of the latter model given both the small sample size (n = 19) and the fact that it predicts a negative fecundity for *H. rubra* less than 120 mm.

There are several possible reasons for the apparent lack of a relationship between fecundity and shell length reported here. These include: spontaneous spawnings during the conditioning period (thereby reducing the number of eggs that could be released when induced to spawn), partial spawning at an induction (whereby only the most mature eggs are released) or the narrow size range (100–130 mm) of the broodstock used. In the case of partial spawners, residual eggs may have either been absorbed or released during subsequent (planned or unplanned) spawning events.

3.5.3 Spawning response time

Mean spawning response times were similar for both sexes of *H. rubra* (6h 16min for males and 6h 29min for females). These times are consistent with the report by Hone, Madigan & Fleming (1997), but less than for Plant et al. (2002). However, Plant et al. (2002) recorded spawning until 02:00h the next morning. Had we continued our observations until that time (rather than 22:00h) the mean spawning response times may have been greater. Spawning response times for *H. laevigata*

were approximately 2.5 h longer than for *H. rubra*. Female *H. laevigata* took longer to respond than males, as seen previously by Plant (2002).

Kikuchi & Uki (1974c) found that the mean spawning response time of *H. discus hannai* was inversely related to the intensity of the UV induction stimulus. Males spawned after 5h 39min and females after 6h 45min when the stimulus was set at 96 mWh/L but these figures declined to 2h 42min and 3h 18min, respectively, when the stimulus was increased to 803 mWh/L. Assuming there were no significant changes in the output of our UV lamps, the intensity of the stimulus (although unknown) was kept constant during our study by maintaining the flow (at 300 ml min^{-1}) to each tray at each induction. Despite this, variations in the spawning response times for both species exceeded those recorded by Kikuchi & Uki (1974c).

Altering the time when induction commences, relative to the photoperiod, can also affect spawning response times in abalone. Uki & Kikuchi (1982b) showed that spawning response times of *H. discus hannai* can be reduced to 1h 20min for females and 1h 45 min for males by supplying UV-irradiated seawater to the broodstock one hour before the onset of the dark phase. Despite the obvious advantages of photoperiod manipulation, which ensures spawning during conventional work hours, few (if any) Australian abalone hatcheries employ this method to alter the timing of spawning. This may be due to the variability in spawning response times of local species, or perceived disruptions to husbandry practices. A systematic evaluation of spawning response times, relative to the application time of the UV stimulus and onset of the dark phase, should be undertaken on *H. rubra* and *H. laevigata* to determine the effectiveness of phase shifted spawning inductions.

3.5.4 Conclusions

Both the mean and total gamete production were higher for male and female *H. rubra* and *H. laevigata* cultured at 16ºC than at 18ºC. The optimal conditioning interval for blacklip abalone at this temperature is ≥1540 EATºC-days (188 days) for males and ≥1350 EATºC-days (165 days) for females. Corresponding figures for *H. laevigata* are ≥1700 EATºC-days (188 days) for males and ≥1930 EATºC-days (212 days) for females. There appears to be considerable flexibility in the duration that blacklip and greenlip abalone may be conditioned on formulated feeds to yield acceptable spawning performance for hatchery production. In most cases, males and females of both species produced large numbers of gametes when induced to spawn following both the first and second conditioning intervals. Conditioning either species at 16ºC and entraining them to a spawning cycle based on the recommended EAT conditioning intervals would allow hatcheries to consistently produce large numbers of gametes for use in seed production (or discarded when the spawning cycle fell between production periods). The groups of animals may be held in rotation while the size and number of groups, and how they are staggered for induction, will be a matter for hatchery management. This study indicates that seedstock can be produced from the hatchery at any time of the year when broodstock are held under favorable culture conditions and provided a formulated feed. Additional work on broodstock nutrition, preferably over two or more conditioning intervals, should be undertaken to determine if spawning performance of these species can be further improved.

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Chapter 4 Lipid and fatty acid composition of pre- and post-spawning blacklip (*Haliotis rubra*) and greenlip (*H. laevigata*) abalone conditioned at two temperatures on a formulated feed.

4.1 Abstract

Wild-caught blacklip (*Haliotis rubra*) and greenlip (*H. laevigata*) abalone fed a formulated feed were held from spent to gravid condition at 14ºC or 18ºC. One third of the gravid animals were induced to spawn (using heated UV-irradiated seawater) and the remainder left untreated. All animals were then killed and samples of foot, digestive gland (DG) and gonad analysed for lipid and fatty acid (FA) composition, as was the feed. The feed contained 5% lipid, of which the major FA were 18:2n-6 (27%), 18:1n-9 (20%) and 16:0 (16%). The lipid content of each tissue was similar for both species, ranging from 4–6%, 8–9%, 14–15%, and 30–32% in the foot, testis, DG and ovary, respectively. Each tissue had a different FA signature, with the foot, testis and ovary characterized by elevated levels of 20:4n-6 (arachidonic acid – ARA), 20:5n-3 (eicosapentaenoic acid – EPA) and 18:2n-6 (linoleic acid – LA), respectively. The proportions of LA and EPA in the DG were intermediate between those of the testis and ovary. There was no change in the lipid or fatty acid composition of blacklip or greenlip tissues in response to the two culture temperatures. Likewise, these compositions did not appear to differ between tissues from spent and gravid individuals. Tissue FA profiles from abalone fed a formulated feed are compared to those from macroalgal feeding trials to determine if the formulated feed can be further improved.

4.2 Introduction

Physical changes in gonad (particularly ovarian) microstructure during gametogenesis of blacklip (*Haliotis rubra*) and greenlip (*H. laevigata*) abalone are well documented (Shepherd and Laws, 1974; Lleonart, 1992; Grubert and Ritar, 2004). However, with the exception of Litaay and De Silva (2003) on female *H. rubra*, there are no works on tissue biochemistry during reproductive development of these species. The gonad, being the site of gametogenesis, is the most important tissue, but the digestive gland and foot also have significant roles, serving as lipid and carbohydrate stores, respectively (Mercer et al., 1993).

There is an increasing body of work which suggests that lipids and fatty acids (FA) play important roles in the growth and gametogenesis of abalone (Webber, 1970; Uki et al., 1986; Bautista-Teruel et al., 2001). Haliotids cannot synthesize all the FA required for normal cellular function and growth (Uki et al., 1986), and rely on dietary sources of these essential fatty acids (EFA) to fulfil their requirements. Restricting the intake, either through reduced feed rations or provision of feeds low in EFA, results in suboptimal growth of abalone (Uki et al., 1986; Floreto et al., 1996; Mai et al., 1996; Dunstan et al., 2000).

In several countries where abalone are farmed, economic and/or ecological concerns regarding the collection of macroalgae for abalone culture have led to the development of formulated feeds. These feeds are usually composed of a mixture of animal and plant products, and as such have very different FA profiles to that of macroalgae. Hence, recent works have compared the effects of natural and formulated feeds on the FA composition of abalone (Dunstan et al., 1996; Su et al., 2004). Others have provided abalone several different manufactured feeds (and in some cases macroalgae as well) and found correlations between feed and tissue FA composition and increased growth (Durazo-Beltrán et al., 2003a,b) or reproductive performance (Bautista-Teruel et al., 2001). As yet, the effect of formulated feeds on the lipid and FA profile of somatic and gonadal tissues from blacklip and greenlip abalone (the main species cultured in Australia) has not been examined.

Diet is not the only factor influencing the FA composition of marine invertebrates. Freezing points of FA are relatively high and inversely related to the degree of unsaturation. Hence, low temperatures may lead to saturated FA freezing, thus reducing membrane fluidity and disrupting membrane function. Several aquatic invertebrates are able to compensate for this by increasing the proportion of unsaturated FA in cell membranes at low temperatures (Cuculescu et al., 1995; Lehti-Koivunen and Kivivuori, 1998; Hall et al., 2002), a phenomenon known as homeoviscous adaptation (Sinensky, 1974). The capacity of abalone to alter their FA profile in response to different temperatures has not been studied.

The primary aim of this work was to describe the lipid and FA composition of selected tissues (i.e. the foot, digestive gland, ovary and testis) from pre- and post-spawning blacklip and greenlip abalone fed a formulated feed. Comparison of the FA profiles of gravid and spent gonads may then allow a description of the FA important to oogenesis and spermatogenesis. A secondary aim was to determine the effect of two different temperatures (14ºC and 18ºC) on the FA composition of these tissues. Reproductive development of abalone at each temperature was standardized by sampling after a particular effective accumulative temperature (EAT) interval (which was different for each species). The EAT being the cumulative difference between the daily water temperature and the biological zero point, the temperature below which gonad development is arrested (Kikuchi and Uki, 1984). Identifying the FA important to gonad development may aid in formulating more suitable broodstock feeds for these species. Separating the potential effect of temperature from that of diet will assist this process.

4.3 Methods

4.3.1 Collection and inspection of animals

Blacklip and greenlip abalone broodstock were collected by a commercial fisher from West End, Settlement Point and Roydon Island (Furneaux group, north-east Tasmania) on 23 November 2000 and 27 April 2001. Animals were transferred to

the Tasmanian Aquaculture and Fisheries Institute, Marine Research Laboratories and held in 600 L tanks supplied with ambient temperature seawater until examination and allocation to experimental treatments. Prior to the start of the experiment (10 May 2001), animals were measured, weighed, tagged and induced to spawn using heated UV-irradiated seawater (Grubert and Ritar, 2002). Only animals that had recently spawned or showed no sign of gonad development when first examined were used in the experiment.

4.3.2 Experimental design

Abalone of each sex and species were held in separate 150 L fibreglass tanks. Within each sex and species group, three animals were assigned to each experimental temperature (i.e. 14ºC or 18ºC), giving 2 x 2 x 2 = 8 tanks in total. Eighteen degrees Celsius was chosen as it represents the maximum temperature typically used to condition *H. rubra* and *H. laevigata* (Fleming, 2000) and 14ºC as gonad development is considerably slower at this temperature (Grubert and Ritar, 2004). Blacklip abalone were conditioned for 1450 EATºC-days (equal to 229 and 145 days at 14ºC and 18ºC, respectively) and greenlip abalone for 1800 EATºC-days (equal to 255 and 165 days at 14ºC and 18ºC, respectively). These effective accumulative temperature (EAT) intervals were chosen as they approximate the optimal conditioning period for each species (Grubert and Ritar, 2005), the EAT being the cumulative difference between the daily water temperature and the biological zero point (which is 7.8ºC in blacklips and 6.9ºC in greenlips; Grubert and Ritar, 2004). When the interval for each species and temperature group had elapsed, two animals of each sex were killed for lipid and FA analysis. At the same time, one male and one female from each group were transferred to spawning trays and spawned using heated UV-irradiated seawater. These animals were killed and sampled for lipid and FA analysis within two days of spawning.

4.3.3 Husbandry and monitoring

Each experimental temperature was maintained using a 10kW heat-chill unit, with flow rate to each tank set at 1.5 L min^{-1}. Animals were fed daily to satiation on a broodstock conditioning feed (Adam and Amos Abalone Foods Pty Ltd). One gram of each batch of feed (n = 5) was frozen for biochemical analysis. Photoperiod was maintained at 12L:12D starting at 06:00h and light intensity at the bottom of the tanks (when filled) was 90–100 Lux. Water temperature was recorded using StowAway TidbiT temperature loggers (Onset Computer Corporation, MA, USA).

4.3.4 Removal and preparation of tissue samples

Abalone were shucked and approximately 1 g of muscle tissue excised from the anterior of the foot and weighed (± 0.001 g). The conical appendage was removed by a transverse cut behind the spire. A second (transverse) cut was made midway between the apex of the spire and the tip of the conical appendage. The distal section was retained for a concurrent histological study and the proximal section for biochemical analysis. Foot and conical appendage samples were frozen in liquid nitrogen then transferred to a freezer at -18ºC. The conical appendage was later partially thawed to allow removal of the gonad from the digestive gland. These tissues were then weighed and refrozen. All samples were freeze dried and reweighed prior to lipid extraction.

4.3.5 Lipid and fatty acid analysis

Feed and tissue samples were quantitatively extracted for lipids using a modification of the method by Bligh and Dyer (1959). Each sample was extracted overnight (methanol and chloroform) and phases separated the following day by addition of chloroform and water (final solvent ratio, 1:1:0.9 v/v/v, methanol/chloroform/water). The total solvent extract was concentrated (i.e. solvents removed in vacuo) using rotary evaporation at 40ºC and lipid content determined gravimetrically.

Fatty acid methyl esters (FAME) were produced from an aliquot of total solvent extract treated with methanol/hydrochloric acid/chloroform (10:1:1 by vol, 80ºC for 2 h; Christie, 1982) and extracted into hexane/chloroform (4:1 v/v, 1 x 1.5 ml). Gas chromatographic (GC) analyses of FAME were performed with a Hewlett Packard 5890A GC equipped with a HP-5 cross-linked (5% Phenyl)-methylpolysiloxane fused silica capillary column (50 m long, 0.32 mm i.d., 0.17µm film thickness), a flame ionization detector (at 310ºC), a split/splitless injector (at 290ºC) and an HP 7673A auto injector. Helium was the carrier gas and samples were injected in splitless mode at an oven temperature of 50ºC. After 1 min, the oven temperature was raised to 210ºC at 30ºC min^{-1}, then to 280ºC at 3ºC min^{-1}. This temperature was maintained for further 5 min. Peaks were quantified with Waters Millennium software (Milford, MA, USA).

Individual components were identified by comparing retention time data with those obtained for authentic and laboratory standards, and selected samples analysed by gas chromatograph-mass spectrometry (GC-MS) to verify FAME identifications. GC-MS analyses of the FAME were performed with a Finnigan GCQ Plus GC-MS System fitted with on-column injection set at 45ºC. Samples were injected with an AS2000 autosampler into a retention gap attached to a HP 5 Ultra2 50 m, 0.32 mm id, and 0.17µm film thickness column using helium for the carrier gas. Mass spectrometer operating conditions were as follows: EV, 70eV; Emission current 250, transfer line 310ºC, source temperature 240ºC, 0.8 $scans^{-s}$ and mass range 40–650 Dalton.

4.4 Results

4.4.1 Analysis of formulated feed

Mean total lipid (± S.E.) and moisture content of the formulated feed was 4.8 ± 1.6% (of dry weight - DW) and 7.7 ± 1.2%, respectively. Linoleic acid (LA, 18:2n-6) constituted 27% of total FA and oleic acid (18:1n-9) 20% (Table 4.1). Other prominent FA included palmitic (16:0; 16%), docosahexaenoic (DHA, 22:6n-3; 6%), eicosapentaenoic (EPA, 20:5n-3; 4%), stearic (18:0; 4%) and

gadoleic (20:1n-9; 3%) acids. The ratio of n-3:n-6 polyunsaturated FA (PUFA) was 0.4:1 and that of arachidonic acid (ARA) to EPA 0.1:1. The ratio of saturated FA (SFA), monounsaturated FA (MUFA) and PUFA was approximately 2:3:4.

4.4.2 Analysis of abalone tissues

Within each sex and species, there was little difference in the lipid or moisture content of each tissue between the different treatments groups (i.e. temperature, pre- and post-spawning). These data were pooled and are presented in Table 4.2. Total lipid in the foot and digestive gland (DG) were similar across species and sexes, but there was a three-fold difference in lipid content between the testis and ovary of both species. In general, the moisture content of male tissues was less than that of female tissues, except in the gonad where the reverse was true.

Mean tissue-specific FA composition (as % of total FA) was relatively consistent among treatment groups for both sexes and species (Tables 4.3–4.6). The most abundant FA in the diet, LA, only formed a minor component (< 6%) of the FA fraction in the foot and testis in both species, but was more common in the DG (12–18%) and ovary (18–26%). The variation in LA was largely responsible for the difference in n-3:n-6 ratios between tissues. Palmitic acid was the major FA in most tissues. Also common were 17:0, 18:0, 18:1n-7, 18:1n-9, 20:1n-9/20:2 non methylene interrupted (NMI) and 22:2 NMI. In both species, the proportion of ARA was higher in the foot than in the DG or gonad (Figs. 1 and 2), resulting in a relatively high ARA:EPA ratio in the foot. The magnitude of this difference was greater in males, as the testis contained approximately ten times more EPA than the ovary. In blacklip abalone, the proportion of DHA in the foot of females was greater than that in the ovary, whereas the reverse was true in corresponding male tissues. DHA was also more abundant in the testis than the foot of male greenlip abalone.

Table 4.1 Percentage fatty acid composition (% of total FA; mean ± S.E; n = 5) of the formulated feed.

Fatty acid	% in Feed
14:0	1.2 ± 1.1
15:0	0.1 ± 0.1
16:0	15.9 ± 1.6
17:0	0.2 ± 0.2
18:0	3.5 ± 1.7
Sum SFA	21.0 ± 1.7
16:1(n-7)	1.6 ± 2.4
16:1(n-5)	2.1 ± 1.5
18:1(n-9)	19.8 ± 1.2
18:1(n-7)	0.7 ± 0.7
20:1(n-9) [a]	3.4 ± 1.8
Sum MUFA	28.0 ± 3.5
18:2(n-6) LA	27.4 ± 2.2
20:3(n-6)	0.0 ± 0.0
20:4(n-6) ARA	0.4 ± 0.2
20:4(n-3)	0.0 ± 0.0
20:5(n-3) EPA	4.1 ± 0.2
22:4(n-6)	0.0 ± 0.0
22:5(n-3)	1.5 ± 0.4
22:6(n-3) DHA	6.1 ± 0.6
22:2 NMI	–
Sum PUFA	39.9 ± 1.8
Other [b]	11.1
Total n-3	11.7 ± 1.0
Total n-6	28.2 ± 2.0
n-3:n-6	0.4
ARA:EPA	0.1

[a] includes 20:2 non-methylene interrupted (NMI) diunsaturated FA.

[b] includes small (< 1%) amounts of: 12:0, 13:0, 14:1, 4,8,12 Trimethyltridecanoic acid (TMTD), 16:1(n-9), branched chain (*bc*)17:1, 17:1, iso (*i*)17:0, anti-iso (*a*)17:0, 18:4(n-3), 18:1(n-5), 20:1(n-7) and 22:5(n-6).

Table 4.2 Mean (± S.E) lipid (% of DW) and moisture (% of WW) content in the foot, digestive gland and gonad of male and female blacklip and greenlip abalone. n = 6, data pooled over temperature and spawning status.

		Blacklip		Greenlip	
Tissue		Female	Male	Female	Male
Foot	Lipid	6.1 ± 0.8	5.0 ± 0.5	4.6 ± 0.6	4.3 ± 0.4
	Moisture	74.3 ± 1.1	70.4 ± 3.7	77.4 ± 2.6	77.2 ± 1.7
Digestive gland	Lipid	13.7 ± 1.3	14.2 ± 1.2	14.6 ± 1.7	13.7 ± 0.8
	Moisture	69.4 ± 1.2	67.6 ± 1.8	72.3 ± 2.4	69.2 ± 1.8
Gonad	Lipid	32.2 ± 2.1	9.2 ± 0.6	29.6 ± 1.5	8.2 ± 0.9
	Moisture	65.2 ± 1.2	69.3 ± 5.2	66.8 ± 1.6	73.6 ± 1.3

Table 4.3 Percentage fatty acid composition (% of total FA; mean ± S.E.; n = 2) of the foot, digestive gland and ovary of spent (EATºC-d = 0) and gravid (EATºC-d = 1450) female blacklip abalone conditioned at two temperatures. Comb. (Combined) = one sample from each temperature.

	Foot			Digestive gland			Ovary		
	0	1450		0	1450		0	1450	
Fatty acid	Comb.	14ºC	18ºC	Comb.	14ºC	18ºC	Comb.	14ºC	18ºC
14:0	0.9±0.9	0.1±0.1	0.9±0.7	2.3±0.9	1.5±1.4	1.3±0.8	6.5±0.2	6.2±0.8	5.1±3.6
15:0	0.6±0.6	0.6±0.0	1.0±1.0	0.3±0.3	0.3±0.3	0.3±0.3	0.6±0.2	0.3±0.0	0.3±0.3
16:0	15.2±2.8	10.7±1.1	15.3±4.0	16.6±0.0	15.3±3.8	18.0±1.9	24.3±0.5	20.7±2.0	19.6±0.1
17:0	7.0±0.3	3.6±0.4	4.4±0.7	2.6±0.1	2.1±0.5	3.5±0.3	0.5±0.1	1.5±0.8	2.2±0.1
18:0	8.8±2.9	6.7±0.5	6.9±1.4	4.0±0.7	4.9±0.3	5.6±1.1	2.7±0.3	2.9±0.2	2.9±0.6
Sum SFA	32.5±1.7	21.7±0.9	28.5±4.9	25.7±0.4	24.1±4.7	28.7±1.6	34.6±1.1	31.5±2.2	30.0±3.4
16:1(n-7)	0.3±0.3	–	0.8±0.8	0.2±0.2	0.2±0.2	1.5±1.5	5.6±0.2	2.1±2.1	4.5±4.5
16:1(n-5)	0.5±0.5	1.2±0.0	0.8±0.8	2.8±0.7	2.1±1.0	0.9±0.9	0.8±0.1	2.8±2.2	2.6±1.0
18:1(n-9)	8.5±1.0	10.5±1.5	8.9±1.3	10.6±0.3	12.4±1.2	12.1±1.8	18.1±0.2	14.1±1.9	16.2±0.3
18:1(n-7)	9.0±1.5	7.5±0.5	7.3±0.4	7.5±0.2	7.3±0.8	8.5±1.0	–[a]	–[a]	–[a]
20:1(n-9)[b]	3.8±0.2	4.8±0.8	4.4±0.7	5.4±1.4	6.4±0.1	7.3±0.5	5.9±0.1	6.5±0.0	6.3±0.9
Sum MUFA	23.5±1.0	24.6±2.0	22.9±0.2	27.2±2.2	28.8±1.5	30.9±2.8	31.4±0.1	26.3±2.1	30.6±2.3
18:2(n-6) LA	3.1±1.2	4.0±0.3	3.6±0.0	11.8±1.6	12.7±1.0	12.6±0.8	21.4±1.4	25.7±4.1	20.8±5.4
20:3(n-6)	0.4±0.4	0.8±0.1	1.2±0.6	2.5±1.0	4.0±0.4	3.6±0.1	1.1±0.3	1.4±0.2	1.2±0.1
20:4(n-6) ARA	6.6±0.3	8.1±0.7	7.6±1.1	1.8±0.2	1.5±0.7	1.2±0.2	1.0±0.2	0.8±0.0	0.7±0.1
20:4(n-3)	0.1±0.1	0.4±0.0	1.1±0.8	0.5±0.5	0.6±0.3	1.0±0.2	0.5±0.3	0.7±0.0	0.9±0.2
20:5(n-3) EPA	3.9±0.9	6.1±0.2	7.0±0.6	3.9±2.3	2.3±0.5	2.6±0.4	1.8±0.3	1.9±0.3	1.9±0.2
22:4(n-6)	2.6±0.1	2.7±0.1	1.6±0.1	0.2±0.0	0.2±0.1	–	0.1±0.1	0.2±0.0	–
22:5(n-3)	8.2±1.7	9.9±1.7	6.9±0.2	1.5±0.9	1.0±0.3	1.1±0.1	1.0±0.2	1.0±0.2	1.0±0.1
22:6(n-3) DHA	0.7±0.7	3.4±1.8	3.0±1.7	0.8±0.4	0.7±0.0	0.9±0.3	0.5±0.1	0.7±0.1	0.7±0.0
22:2 NMI	5.1±0.9	6.7±0.3	5.0±0.2	4.8±1.0	5.9±1.4	6.5±0.4	3.2±0.3	3.3±0.2	3.6±0.3
Sum PUFA	30.9±6.3	42.4±0.6	37.2±0.7	27.8±6.9	29.6±2.2	29.5±0.7	30.7±2.1	35.9±5.3	31.0±4.6
Other[c]	13.1	11.3	11.4	19.4	17.5	10.9	3.3	6.3	8.4
Total n-3	13.0±3.5	19.8±0.3	18.0±0.6	6.7±3.1	4.6±1.1	5.6±1.0	3.8±0.9	4.3±0.6	4.4±0.5
Total n-6	12.8±1.9	15.9±0.7	14.2±1.5	16.3±2.8	19.2±0.3	17.4±0.7	23.6±1.4	28.3±4.5	22.9±5.5
n-3:n-6	1.0	1.2	1.3	0.4	0.2	0.3	0.2	0.2	0.2
ARA:EPA	1.7	1.3	1.1	0.5	0.7	0.5	0.6	0.4	0.4

[a] could not be determined as peak for 18:1(n-7) co-eluted with that for 18:1(n-9); [b] includes 20:2 NMI; [c] includes small (< 1%) amounts of: 12:0, 13:0, 14:1, 4,8,12 TMTD, 16:1(n-9), *bc*16:0, *i*17:0, *a*17:0, 18:4(n-3), 18:1(n-5), 19:0, 20:1(n-7), 20:0, 21:5(n-3) and 22:5(n-6).

Table 4.4 Percentage fatty acid composition (% of total FA; mean ± S.E.; n = 2) of the foot, digestive gland and testis of spent (EATºC-d = 0) and gravid (EATºC-d = 1450) male blacklip abalone conditioned at two temperatures. Comb. (Combined) = one sample from each temperature.

	Foot			Digestive gland			Testis		
	0	1450		0	1450		0	1450	
Fatty acid	Comb.	14ºC	18ºC	Comb.	14ºC	18ºC	Comb.	14ºC	18ºC
14:0	1.6±1.2	1.6±0.3	1.7±1.5	0.5±0.5	2.6±0.1	2.6±0.4	1.7±1.5	0.7±0.0	0.9±0.2
15:0	1.1±1.1	1.7±0.1	1.3±1.3	2.2±0.2	0.7±0.1	0.9±0.3	1.6±0.3	1.5±0.6	1.1±0.3
16:0	15.2±2.8	17.9±0.5	13.9±6.9	19.3±0.4	17.0±1.3	18.9±0.4	18.6±2.0	16.2±0.2	19.6±0.1
17:0	4.2±1.4	6.6±2.8	2.7±0.9	3.8±1.9	1.3±1.3	3.1±0.4	2.2±0.1	7.0±0.7	3.8±0.1
18:0	6.8±0.3	6.4±0.2	5.0±5.0	5.6±1.2	3.7±0.2	4.4±0.2	3.5±0.2	4.8±0.4	4.9±0.3
Sum SFA	29.0±6.2	34.1±3.0	24.5±1.2	31.5±3.8	25.3±0.2	29.9±0.9	27.6±3.0	30.1±0.5	30.2±0.1
16:1(n-7)	0.9±0.9	–	–	–	0.6±0.6	2.0±0.9	0.3±0.3	–	0.3±0.3
16:1(n-5)	–	2.9±0.0	2.5±2.5	–	2.4±0.6	0.8±0.8	0.3±0.3	0.3±0.3	1.1±0.0
18:1(n-9)	9.3±0.8	9.7±0.1	12.6±5.5	9.7±0.8	10.2±1.3	9.6±1.0	3.8±0.0	4.2±0.1	6.3±0.3
18:1(n-7)	7.3±1.7	9.3±1.6	8.9±1.1	6.6±0.2	5.1±0.2	5.6±0.2	8.9±3.5	9.5±0.8	7.6±0.3
20:1(n-9) [a]	4.1±1.6	8.1±4.6	4.8±1.4	6.6±0.7	6.3±0.6	6.2±0.5	7.3±0.5	5.6±0.8	8.5±0.9
Sum MUFA	22.3±0.1	31.0±7.4	35.4±8.1	23.0±0.2	24.8±0.7	24.5±1.9	21.5±3.1	19.9±0.4	24.8±0.0
18:2(n-6) LA	2.1±0.4	3.8±0.7	5.8±2.4	14.9±0.5	17.7±0.7	13.0±1.1	2.9±0.5	3.6±0.4	5.0±0.8
20:3(n-6)	0.9±0.5	0.6±0.3	0.6±0.3	2.4±2.4	2.9±0.7	3.6±0.1	3.2±0.9	4.7±1.2	4.6±0.1
20:4(n-6) ARA	6.5±0.3	5.6±0.4	4.1±1.6	2.5±0.5	0.8±0.4	1.0±0.1	1.2±0.1	1.3±0.1	1.2±0.1
20:4(n-3)	0.6±0.2	1.1±0.8	0.3±0.3	0.3±0.3	3.1±2.0	0.4±0.0	1.3±0.0	2.8±0.1	2.8±0.2
20:5(n-3) EPA	3.9±0.1	3.3±0.8	2.7±0.8	3.7±0.9	1.4±0.5	2.5±0.4	15.4±2.0	18.3±3.3	14.6±0.1
22:4(n-6)	2.9±0.2	2.0±0.1	1.7±0.9	0.2±0.2	0.1±0.1	0.1±0.0	0.3±0.0	0.1±0.1	0.1±0.1
22:5(n-3)	8.1±1.4	5.6±0.9	5.1±2.4	1.7±0.1	0.8±0.1	0.9±0.1	2.0±0.4	4.1±0.6	3.6±0.2
22:6(n-3) DHA	0.7±0.4	1.2±0.6	1.2±0.0	0.9±0.1	0.6±0.0	0.7±0.1	0.9±0.1	1.9±0.3	2.4±0.2
22:2 NMI	6.2±1.3	3.9±0.4	3.9±1.7	7.8±0.3	5.4±0.5	5.0±0.1	3.0±0.1	2.2±0.1	2.8±0.0
Sum PUFA	31.8±3.8	27.2±5.0	25.9±4.6	34.5±1.2	33.2±1.0	27.6±1.0	30.0±2.9	39.0±3.3	37.4±0.6
Other [b]	16.9	7.7	14.2	10.9	16.7	18.0	20.8	11.0	7.6
Total n-3	13.3±1.8	11.2±3.1	9.3±2.9	6.5±0.9	5.9±1.6	4.6±0.4	19.5±2.6	27.0±4.3	23.4±0.7
Total n-6	12.4±0.7	12.0±1.4	12.7±0.0	20.2±1.8	22.0±1.1	18.1±1.2	7.5±0.4	9.7±0.9	11.2±1.3
n-3:n-6	1.1	0.9	0.7	0.3	0.3	0.3	2.6	2.9	2.1
ARA:EPA	1.7	1.7	1.5	0.7	0.6	0.4	0.1	0.1	0.1

[a] includes 20:2 NMI; [b] includes small (< 1%) amounts of: 12:0, 13:0, 14:1, 4,8,12 TMTD, 16:1(n-9), *bc*16:0, *i*17:0, *a*17:0, 18:4(n-3), 18:1(n-5), 19:0, 20:1(n-7), 20:0, 21:5(n-3) and 22:5(n-6).

Table 4.5 Percentage fatty acid composition (% of total FA; mean ± S.E.; n = 2) of the foot, digestive gland and ovary of spent (EATºC-d = 0) and gravid (EATºC-d = 1800) female greenlip abalone conditioned at two temperatures. Comb. (Combined) = one sample from each temperature.

	Foot			Digestive gland			Ovary		
	0	1800		0	1800		0	1800	
Fatty acid	Comb.	14ºC	18ºC	Comb.	14ºC	18ºC	Comb.	14ºC	18ºC
14:0	0.8±0.8	0.7±0.7	0.7±0.1	1.6±0.3	1.9±0.4	2.2±0.3	3.3±1.3	5.6±1.1	5.6±0.7
15:0	1.4±0.1	1.5±0.5	0.5±0.5	0.7±0.3	0.9±0.3	0.7±0.0	0.7±0.0	0.3±0.0	0.3±0.0
16:0	10.8±7.7	12.8±4.3	14.9±1.1	16.5±3.3	20.5±2.4	18.6±1.4	20.4±1.4	20.1±0.9	23.6±3.0
17:0	6.4±0.1	5.7±0.6	8.7±2.8	2.7±1.1	4.1±1.1	1.9±0.3	2.6±0.6	1.5±0.6	1.2±0.5
18:0	6.0±0.7	6.2±0.7	7.0±1.1	4.2±0.4	5.4±1.0	3.6±0.0	5.0±2.3	2.2±0.0	2.5±0.1
Sum SFA	25.5±9.2	26.9±4.2	31.9±2.3	25.8±5.3	32.8±5.1	27.1±1.9	32.0±0.2	29.7±0.4	33.2±3.2
16:1(n-7)	–	–	–	–	–	0.9±0.4	3.1±3.1	4.5±4.5	3.3±3.3
16:1(n-5)	1.2±1.2	1.3±0.7	1.3±0.1	3.0±0.4	2.6±0.2	2.8±0.7	1.8±1.1	3.9±3.1	3.3±2.7
18:1(n-9)	6.3±1.0	9.4±2.2	10.1±1.9	8.2±0.2	11.3±0.4	9.7±0.0	17.9±0.0	15.3±2.7	14.4±0.1
18:1(n-7)	5.5±2.2	7.0±0.3	7.6±0.1	6.6±0.1	6.3±0.1	6.9±0.4	–[a]	–[a]	–[a]
20:1(n-9)[b]	4.0±0.9	3.7±1.2	5.2±0.3	7.1±0.2	7.4±1.3	8.2±1.1	6.6±0.8	4.3±0.2	5.9±0.0
Sum MUFA	17.0±3.3	21.3±2.4	24.1±2.4	25.5±0.1	27.8±1.4	28.8±1.7	30.3±2.8	28.4±1.4	27.6±0.5
18:2(n-6) LA	2.5±0.9	3.3±1.0	3.8±0.0	13.1±0.7	13.5±0.8	16.3±3.7	18.1±0.0	19.7±0.4	19.7±1.2
20:3(n-6)	0.7±0.1	0.9±0.3	0.5±0.5	0.7±0.3	0.9±0.4	0.7±0.1	1.7±0.1	1.3±0.0	1.1±0.1
20:4(n-6) ARA	5.6±0.6	7.7±0.1	5.8±0.0	1.4±0.4	1.2±0.4	1.0±0.4	0.9±0.2	1.0±0.1	0.9±0.1
20:4(n-3)	0.2±0.2	0.8±0.2	0.7±0.7	2.4±0.2	0.3±0.0	3.5±0.6	1.2±0.0	0.9±0.0	0.7±0.1
20:5(n-3) EPA	5.2±0.0	4.6±1.0	3.8±0.5	4.6±1.4	1.9±0.1	2.5±0.7	4.7±1.8	3.2±0.7	2.5±0.2
22:4(n-6)	2.7±0.2	3.1±0.4	2.6±0.5	0.2±0.1	0.1±0.1	0.2±0.1	0.1±0.1	–	0.2±0.0
22:5(n-3)	10.3±1.6	10.0±1.8	8.6±1.2	2.4±1.1	0.8±0.1	1.5±0.2	2.0±0.0	1.8±0.2	1.9±0.2
22:6(n-3) DHA	1.3±0.3	1.0±0.5	1.1±0.4	1.1±0.3	0.7±0.1	0.7±0.1	0.7±0.1	1.6±0.7	1.1±0.2
22:2 NMI	6.3±2.1	5.3±1.0	5.9±0.7	5.4±0.1	6.1±0.4	5.3±1.2	3.4±1.0	2.8±0.1	3.5±0.2
Sum PUFA	34.8±2.7	36.8±6.3	32.8±2.2	31.8±3.9	28.8±0.8	32.6±1.6	33.2±1.1	32.6±2.0	31.9±2.2
Other[c]	22.6	15.1	11.2	16.9	10.6	11.5	4.5	9.3	7.3
Total n-3	16.9±2.0	16.5±3.4	14.2±1.5	10.4±3.0	3.8±0.2	8.2±0.3	8.7±2.0	7.4±1.5	6.2±0.7
Total n-6	11.6±1.4	15.0±1.9	12.7±0.0	16.0±1.0	19.0±1.1	19.1±3.0	21.2±0.1	22.4±0.4	22.2±1.3
n-3:n-6	1.5	1.1	1.1	0.6	0.2	0.4	0.4	0.3	0.3
ARA/EPA	1.1	1.7	1.5	0.3	0.6	0.4	0.2	0.3	0.3

[a] could not be determined as peak for 18:1(n-7) co-eluted with that for 18:1(n-9); [b] includes 20:2 NMI; [c] includes small (< 1%) amounts of: 12:0, 13:0, 14:1, 4,8,12 TMTD, 16:1(n-9), *bc*16:0, *i*17:0, *a*17:0, 18:4(n-3), 18:1(n-5), 19:0, 20:1(n-7), 20:0, 21:5(n-3) and 22:5(n-6).

Table 4.6 Percentage fatty acid composition (% of total FA; mean ± S.E.; n = 2) of the foot, digestive gland and testis of spent (EATºC-d = 0) and gravid (EATºC-d = 1800) male greenlip abalone conditioned at two temperatures. Comb. (Combined) = one sample from each temperature.

	Foot			Digestive gland			Testis		
	0	1800		0	1800		0	1800	
Fatty acid	Comb.	14ºC	18ºC	Comb.	14ºC	18ºC	Comb.	14ºC	18ºC
14:0	0.6±0.6	1.1±0.0	1.0±0.8	1.7±0.1	1.7±0.1	1.8±0.2	0.6±0.0	0.7±0.3	0.7±0.1
15:0	2.6±0.3	1.7±0.2	1.3±0.7	1.4±0.8	0.7±0.1	1.0±0.0	0.8±0.4	0.6±0.1	0.9±0.1
16:0	15.8±3.4	18.6±0.9	12.9±4.0	19.9±0.1	19.5±2.3	21.7±0.3	17.6±0.8	15.8±0.7	18.7±1.9
17:0	5.0±0.5	6.7±2.9	3.5±0.8	3.0±1.3	3.6±0.3	2.9±0.2	4.2±1.1	3.3±0.6	4.4±2.0
18:0	8.7±2.3	6.5±0.7	6.7±0.9	4.4±0.0	4.8±0.6	4.6±0.2	4.9±0.3	5.1±0.4	5.3±0.7
Sum SFA	32.7±2.4	34.6±3.3	25.4±3.7	30.4±2.1	30.2±2.6	31.9±0.0	28.1±1.1	25.5±1.3	30.1±4.9
16:1(n-7)	–	–	–	–	–	0.7±0.3	–	0.7±0.7	0.5±0.1
16:1(n-5)	0.8±0.8	1.0±1.0	1.4±0.8	2.8±0.1	2.2±0.5	1.7±0.4	0.9±0.2	0.3±0.0	0.6±0.1
18:1(n-9)	6.1±2.5	6.4±0.3	6.5±0.4	10.6±0.1	10.2±0.3	13.4±1.3	6.9±1.0	5.9±1.5	6.9±0.7
18:1(n-7)	6.2±1.9	6.4±0.3	7.1±0.1	7.4±0.4	7.2±0.3	6.2±0.6	9.3±2.0	8.9±0.8	8.6±0.5
20:1(n-9) [a]	3.2±0.2	3.1±0.1	1.9±0.4	8.1±0.9	8.2±1.6	6.8±0.2	6.6±0.6	7.1±0.7	4.0±4.0
Sum MUFA	16.5±5.5	16.9±1.6	17.1±0.5	29.2±0.5	28.1±2.5	29.2±1.4	24.5±2.2	24.1±3.9	24.5±2.0
18:2(n-6) LA	2.7±1.6	3.6±0.1	2.1±0.1	12.4±1.7	12.6±0.3	14.2±0.4	3.6±0.4	3.4±0.6	3.7±0.6
20:3(n-6)	0.6±0.0	0.8±0.0	0.5±0.1	3.6±0.1	1.0±0.1	4.4±0.5	4.2±0.1	4.5±0.4	4.3±0.9
20:4(n-6) ARA	6.8±0.9	6.8±2.3	6.6±0.6	1.5±0.8	1.0±0.1	0.8±0.1	1.1±0.6	0.5±0.1	1.2±0.0
20:4(n-3)	0.9±0.2	0.7±0.3	0.5±0.3	0.2±0.2	4.2±0.2	0.2±0.2	3.7±1.7	5.4±0.6	3.5±0.4
20:5(n-3) EPA	4.0±0.3	5.3±0.2	3.2±0.1	2.2±0.2	1.9±0.1	1.4±0.1	16.9±1.7	17.8±3.1	16.5±2.9
22:4(n-6)	2.6±0.6	2.4±0.4	2.7±0.2	0.2±0.1	–	0.1±0.1	0.2±0.2	–	0.3±0.0
22:5(n-3)	10.4±2.0	9.2±1.3	7.7±0.6	1.2±0.2	1.0±0.2	0.8±0.0	5.2±0.0	5.8±1.1	5.2±0.4
22:6(n-3) DHA	0.4±0.4	1.2±0.2	0.6±0.1	0.5±0.1	0.5±0.0	0.6±0.0	1.7±0.8	2.8±0.2	2.1±0.4
22:2 NMI	5.5±1.0	5.0±0.2	4.2±0.2	4.9±0.2	5.3±0.5	4.0±0.2	2.1±0.4	2.0±0.3	2.5±0.5
Sum PUFA	33.9±2.1	35.1±3.5	28.8±2.2	27.3±0.1	28.1±0.4	26.9±0.6	38.7±3.2	42.1±4.5	39.3±2.3
Other [b]	16.8	13.4	28.6	13.1	13.6	12.0	8.6	8.3	6.2
Total n-3	15.7±1.1	16.4±0.6	12.1±0.9	4.1±0.3	7.7±0.1	2.9±0.2	27.5±4.1	31.8±5.0	27.3±4.1
Total n-6	12.7±0.1	13.7±2.7	12.6±1.1	18.4±0.5	15.0±0.9	19.9±0.5	9.2±0.5	8.3±0.3	9.5±1.4
n-3:n-6	1.2	1.2	1.0	0.2	0.5	0.1	3.0	3.9	3.0
ARA/EPA	1.7	1.3	2.0	0.7	0.5	0.6	0.1	0.0	0.1

[a] includes 20:2 NMI; [b] includes small (< 1%) amounts of: 12:0, 13:0, 14:1, 4,8,12 TMTD, 16:1(n-9), *bc*16:0, i17:0, *a*17:0, 18:4(n-3), 18:1(n-5), 19:0, 20:1(n-7), 20:0, 21:5(n-3) and 22:5(n-6).

Figure 4.1 Percentage total fatty acid (TFA; mean ± S.E.) of linoleic (LA, 18:2n-6), arachidonic (ARA, 20:4n-6), eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:6n-3) acids in the foot, digestive gland (DG) and gonad of female (a–c) and male (d–f) blacklip abalone. Data pooled for temperature and spawning state.

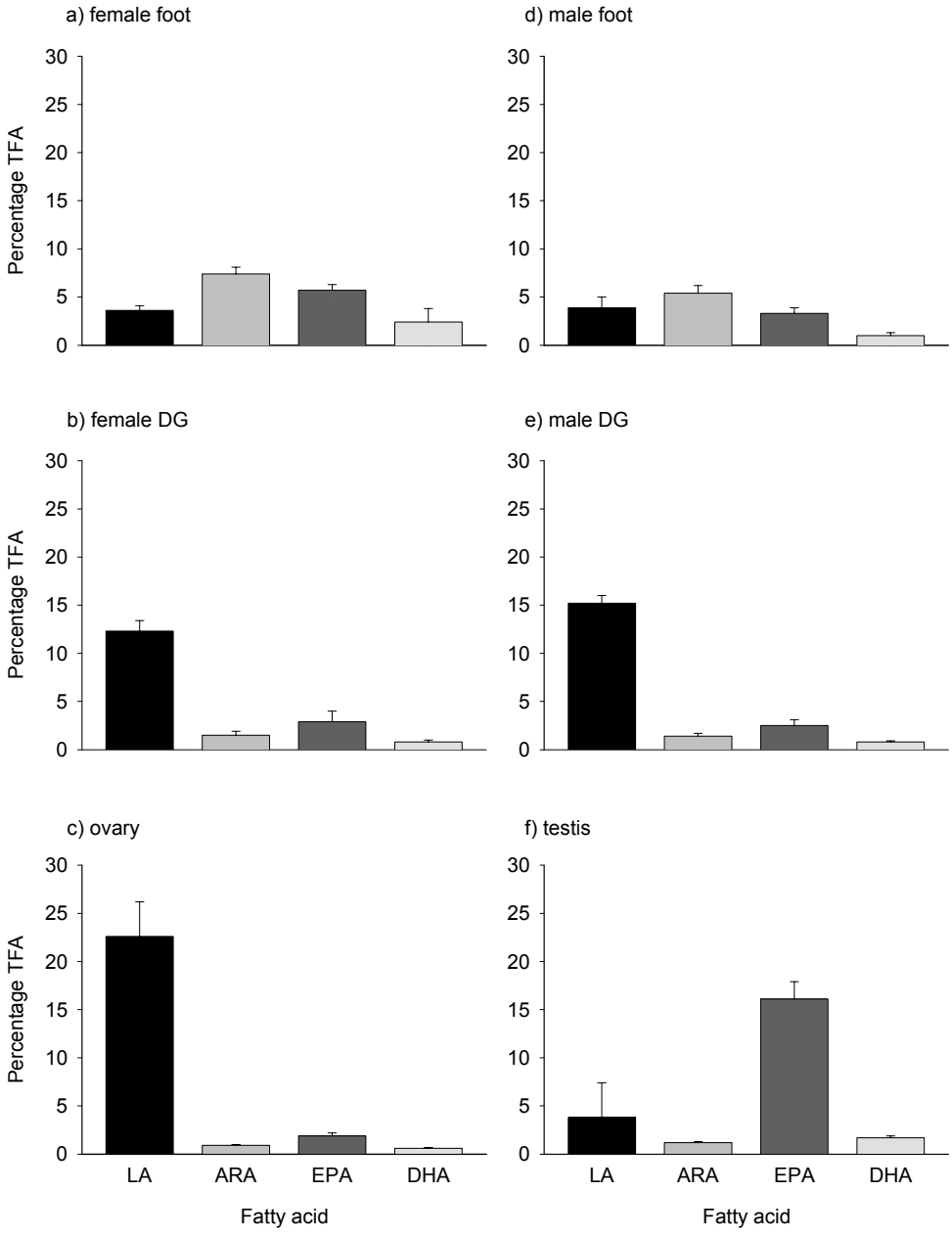
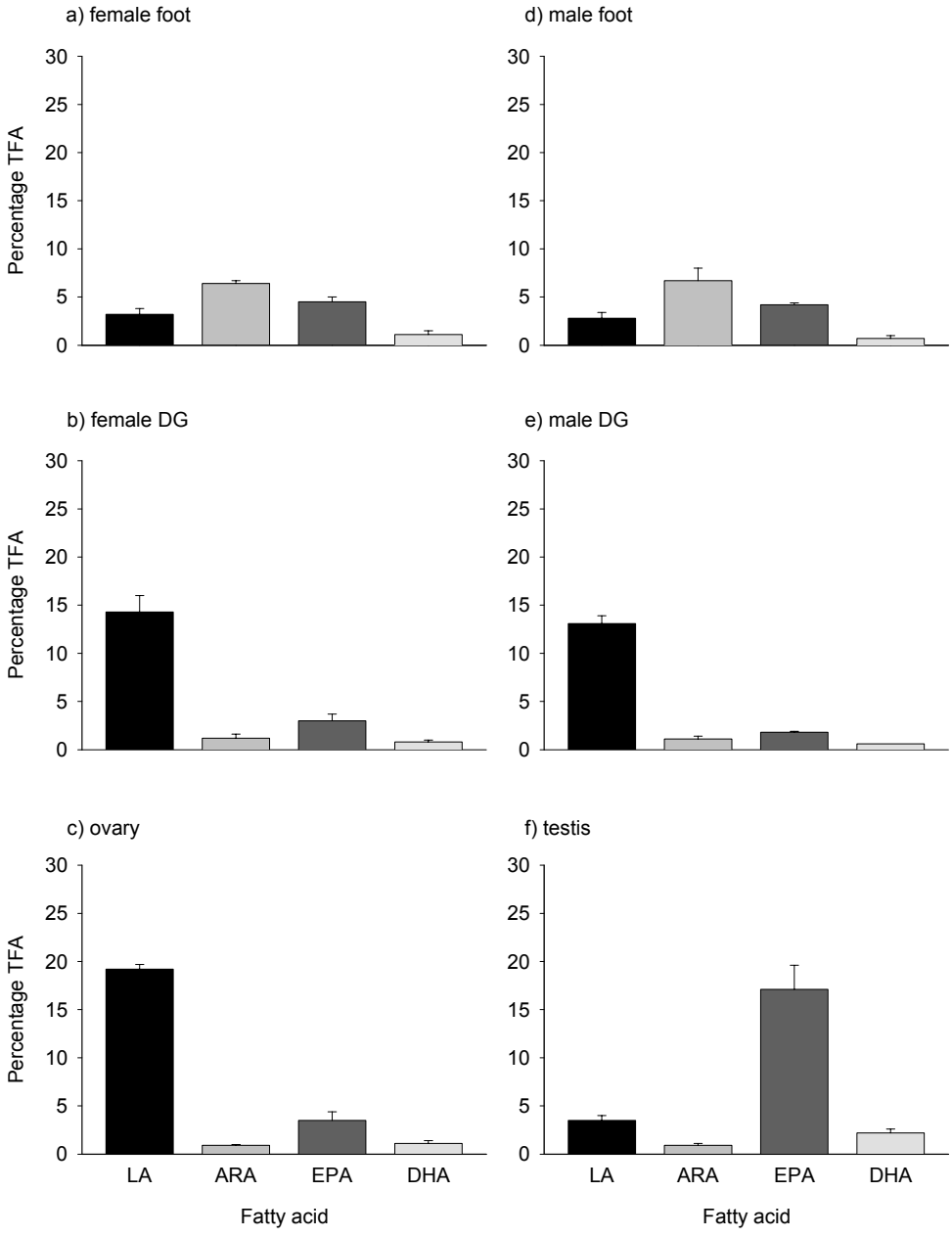


Figure 4.2 Percentage total fatty acid (TFA; mean ± S.E.) of linoleic (LA, 18:2n-6), arachidonic (ARA, 20:4n-6), eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:6n-3) acids in the foot, digestive gland (DG) and gonad of female (a–c) and male (d–f) greenlip abalone. Data pooled for temperature and spawning state.



4.5 Discussion

Total lipid in the foot of both sexes of blacklip (*Haliotis rubra*) and greenlip (*H. laevigata*) abalone were relatively low (4–6% DW), similar to previous reports on both wild and cultured abalone (Webber, 1970; Floreto et al., 1996; Chiou et al., 2001; Nelson et al., 2002a). Digestive gland, testicular and ovarian lipid content showed little variation between spent and gravid animals of either species (14–15%, 8–9% and 30–32% DW, respectively). The lack of variation in DG and ovarian lipid between these groups was unexpected given the wide-ranging values reported for wild *H. rubra* (15–25% and 31–40% DW, respectively; Litaay and De Silva, 2003) and *H. cracherodii* (8–28% and 24–38% DW, respectively; Webber, 1970). Both these works sampled for a year or more and encompassed an entire seasonal cycle of gametogenesis, spawning and resorption of the gonad (into the DG). However, spent animals in this study were sampled within two days of spawning and so may not have resorbed all residual gonad tissue. This may explain the similarities in tissue lipid (and FA) composition between spent and gravid animals.

Abalone require n-3 and n-6 PUFA (such as EPA and ARA) as essential fatty acids (Uki et al., 1986) with the n-3:n-6 ratio providing a broad indication of FA utilisation within tissues. Values of this ratio for a given tissue type were similar for *H. rubra* and *H. laevigata*. In each case, the foot contained equal proportions of both PUFA types, whereas n-6 PUFA's were more prominent in the DG and ovary (and the formulated feed). By contrast, the testis contained 3–10 times more n-3 PUFA than the other tissues.

The formulated feed provided to adult *H. rubra* and *H. laevigata* during this study had higher n-3 PUFA and lower n-6 PUFA composition (primarily LA) than that fed to juvenile *H. laevigata* and *H. laevigata* x *H. rubra* hybrids by Dunstan et al. (1996). Despite this, foot muscle FA profiles were similar between the different groups, although the juveniles contained a greater proportion of both n-3 and n-6 PUFA than adults. By contrast, there was considerable difference in the

proportions of palmitic acid in the foot of *H. rubra* between this study (11–18% of total FA) and that of Su et al. (2004; 57% of total FA) even though both groups were fed a similar feed. The extremely high proportion of palmitic acid in the samples analysed by Su et al. (2004) was offset by lower proportions of 18:1n-9, 18:1n-7 and 22:5n-3. The reasons behind these differences are unclear.

Foot muscle FA profiles of *H. rubra* and *H. laevigata* fed a formulated feed show several differences to that of *H. fulgens* fed macroalgae (Nelson et al., 2002a). One is the lower proportion of SFA (primarily 16:0) in the foot of *H. rubra* and *H. laevigata* (~28%) compared to that of *H. fulgens* (39%). Another is the smaller fraction of ARA in the former (~6%) than the latter (12%). Interestingly, blacklip and greenlip abalone collected from the wild contained 11% and 14% ARA, respectively (Dunstan et al., 1996), similar to that of *H. fulgens* fed macroalgae. These figures are also consistent with other works on both wild-caught and captive abalone fed macroalgae (e.g. Kochi, 1975; Floreto et al., 1996).

When provided a formulated feed, the FA profiles of the DG of female *H. rubra* and *H. laevigata* were similar to that of female *H. asinina* (Bautista-Teruel et al., 2001). The proportion of 16:0, LA, ARA and EPA in the DG of all three species ranged from 15–23%, 9–12%, 1–2% and 2–4%, respectively. However, the proportion of 14:0 in the DG of *H. rubra* and *H. laevigata* was ~2% as opposed to 14% in *H. asinina*. This was most probably a dietary effect, as the feed offered here contained only 1% 14:0, whereas that offered to *H. asinina* contained 17% 14:0 (Bautista-Teruel et al., 2001). Another difference was in the proportion of 18:1n-7, which was 6–9% in the DG of *H. rubra* and *H. laevigata* and 19% in the DG of *H. asinina*. Given that the peaks for 18:1n-7 and 18:1n-9 tend to co-elute (pers. obs.) and that Bautista-Teruel et al. (2001) did not report the proportion of the latter, it is probable that the value they gave for 18:1n-7 represents the sum of both it and 18:1n-9. Furthermore, the proportion of 18:1n-7 reported by Bautista-Teruel et al. (2001) approximates the cumulative fraction of 18:1n-7 and 18:1n-9 in the DG of blacklips and greenlips (19% and 16%, respectively).

Several works have described the FA profile of the DG of abalone, but there has been little consistency in tissue terminology between them. For example, the DG was termed the viscera by Kochi (1975), liver by Floreto et al. (1996) and hepatopancreas by Bautista-Teruel et al. (2001) and Nelson et al. (2002a), the hepatopancreas being the major component of the gonad/hepatopancreas complex in the latter study (M. Nelson, pers. comm.). While these studies analysed different species from different environments (i.e. wild vs macroalgal-fed captive animals), there was some consistency in their DG FA profiles. In general, the DG of abalone fed macroalgae contained high (8–30%) proportions of both ARA and EPA and low (1–5%) proportions of LA (Kochi, 1975; Floreto et al., 1996; Nelson et al., 2002a), almost the exact reverse of animals provided formulated feeds. These differences are largely dietary modulated as macroalgae typically contain more ARA and EPA and less LA than formulated feeds (Floreto et al., 1996; Bautista-Teruel et al., 2001; Nelson et al., 2002b; present study).

The FA compositions of the testes of *H. rubra* and *H. laevigata* were similar. The proportions of LA, ARA, EPA and DHA in the testes of both species ranged from 3–5%, 0.5–1%, 15–18% and 1–3%, respectively. In all but one case (ARA), these figures were different to those of the formulated feed as well as the other tissues, suggesting that abalone regulate the FA composition of their testes. Abalone require EPA in the diet rather than DHA (Uki et al., 1986), and when dietary EPA is low (e.g. 2% of total FA in this study) they are able to retroconvert DHA to form EPA (Dunstan et al., 1996). This may explain the differential proportions of DHA and EPA in the testis compared to that in the manufactured feed.

Ovarian FA profiles were similar for temperate *H. fulgens* fed macroalgae (Nelson, 1999) and tropical *H. asinina* from the wild (Bautista-Teruel et al., 2001). In both, there was a narrow range in ovarian 14:0 (11–13%), 18:0 (1–3%), ARA (3–4%) and EPA (3–5%). With the exception of 18:0, these values are greater than in the current study. As for the DG, the major difference between abalone fed formulated and natural feeds was in the proportion of LA, which constituted 18–26% of total FA in the ovary of *H. rubra* and *H. laevigata* and

11% and 1% of that in *H. asinina* and *H. fulgens* (fed macroalgae), respectively (Bautista-Teruel et al., 2001, Nelson, 1999). Similar to female *H. fulgens* (Nelson et al., 2002a), we observed a decline in the proportion of ARA (and the ARA:EPA ratio) between the foot, DG and gonad of female *H. rubra* and *H. laevigata*. Given the skewed ARA:EPA ratio in the ovary and the fact that both FA are prostaglandin precursors (Sargent, 1995) we suggest that ARA is more important to oogenesis of abalone than EPA and that increasing the ARA content of formulated feeds may increase fecundity.

Although the FA composition of the ovary and larvae from *H. fulgens* broodstock fed macroalgae are similar (Nelson, 1999; Nelson et al., 2002a), this does not appear to be the case for *H. laevigata* fed a formulated feed. Greenlip abalone used in both this study and that of Daume and Ryan (2004) were fed the same commercially available broodstock feed. However, the proportion of LA in freshly-spawned ova (1–7%; Daume and Ryan 2004) was lower than in ovarian tissue (19%; this study). Regional variation in FA metabolism is a possible reason behind this difference, as broodstock for each study were taken from sites approximately 3000 km apart. Alternatively, some FA may be concentrated in the supportive tissue, with little or no transfer to the oocytes.

While thermal preference and broodstock conditioning trials show that blacklip and greenlip abalone prefer 18ºC to 14ºC (Gilroy and Edwards, 1998) and mature faster at the higher temperature (Grubert and Ritar, 2005), this difference did not appear to affect the FA profiles of these species, suggesting that tissue FA were maintained at constant/optimum levels. This may also have been due to the relatively small difference between experimental temperatures. Sampling animals held at the extremes of their thermal range (e.g. 10ºC vs 20ºC) may provide a better understanding of the effect of temperature on the FA profile of abalone tissues.

This study showed that the lipid and FA profile of corresponding tissues from blacklip and greenlip abalone fed a formulated feed were similar. Each tissue had a unique FA signature, with the foot, testis and ovary characterized by elevated levels of ARA, EPA and LA, respectively. The proportion of LA and EPA in the DG was intermediate between that of the ovary and testis. The difference between the two experimental temperatures was insufficient to elicit a change in the lipid or FA composition. There were no apparent differences in the lipid or FA profile of gravid and spent individuals, presumably due to partial spawning and/or incomplete resorption of the gonad. Animals preferentially accumulated and/or synthesized ARA from C_{18} precursors, as shown by the greater proportion of ARA in the foot than the formulated feed. Further work, including more intensive post-spawning sampling, needs to be conducted to fully understand the lipid and FA dynamics during gametogenesis of these and other abalone species.

4.6 Acknowledgements

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Chapter 5 The effects of sperm density and gamete contact time on the fertilisation success of blacklip (*Haliotis rubra*; Leach, 1814) and greenlip (*H. laevigata*; Donovan, 1808) abalone

5.1 Abstract

A factorial experiment, in which blacklip (*Haliotis rubra*; Leach, 1814) and greenlip abalone eggs (*H. laevigata*; Donovan, 1808) were exposed to a range of conspecific sperm densities (ca. 10^4–10^7 sperm ml^{-1}) for different time intervals (7–2400 s), showed there was a significant interaction between these factors in both species. Prolonged exposure (i.e. 1200–2400 s for blacklips and 480–2400 s for greenlips) to concentrated sperm (i.e. 10^7 sperm ml^{-1}) resulted in lysis of the egg membrane and polyspermy. Analysis of CoVariance of F_{50} values (i.e. the sperm concentration required for 50% fertilisation, derived from the linear regression of logit (proportion of eggs fertilised) versus sperm density) between species across a range of contact times demonstrated that contact time had a significant effect ($p < 0.001$) whereas species did not ($p = 0.22$). The lack of a species effect suggests that the fertilisation potential of blacklip and greenlip abalone eggs are similar, at least across the range of sperm densities and contact times used. An examination of sperm morphology using scanning electron microscopy of both species revealed similarities in sperm length (i.e. 42–46 μm) and differences in the shape of acrosome, the tip of which was blunt in blacklip sperm and V-shaped in greenlip sperm. Morphological differences in haliotid sperm are discussed in relation to species differences in fertilisation kinetics.

5.2 Introduction

Blacklip (*H. rubra*; Leach, 1814) and greenlip (*H. laevigata*; Donovan, 1808) abalone are fished commercially in waters off southern Australia. They generally mature at around 100 mm (Shepherd and Laws, 1974; Wells and Mulvay, 1992; Tarbath and Officer, 2003) and attain a maximum size of 220 mm (Kailola et al., 1993) although there is considerable variability in these traits, particularly between sites. Their reproductive strategy of broadcast spawning necessitates a high fecundity; female blacklips spawn as many as 5.9×10^6 eggs (Litaay and De Silva, 2001) and greenlips up to 8.2×10^6 eggs (Babcock and Keesing, 1999). Males of both species spawn up to 10^{12} sperm (Babcock and Keesing, 1999; Grubert and Ritar, 2005).

Fertilisation success of free-spawning marine invertebrates is reliant not only on prolific gamete production, but also adult density and synchronicity of spawning. Indeed, separation distances of just two metres between male and female abalone can result in a 45% reduction in fertilisation rate compared to that of adjacent animals (Babcock and Keesing, 1999). In extreme cases, where animals are sparsely distributed, sperm becomes rapidly diluted and the chance of sperm-egg contact is very low, resulting in poor fertilisation success - a phenomenon known as the Allee effect (Allee, 1931).

The optimal sperm density for fertilisation in abalone has been documented for several species (Kikuchi and Uki, 1974; Leighton and Lewis, 1982; Clavier, 1992; Mill and McCormick, 1992; Encena et al., 1998), but few works have examined the combined effects of sperm density and gamete contact time on fertilisation success (see Babcock and Keesing, 1999; Baker and Tyler, 2001). Gamete contact time is most critical at very low and very high sperm densities. Long contact times (40 min) at low sperm densities (10^3 sperm ml^{-1}) are necessary for high (>80%) fertilisation rates (Babcock and Keesing, 1999). Conversely, short contact times (< 15 min) at high (10^7 sperm ml^{-1}) sperm densities lessen (or eliminate) the effect of polyspermy (Ebert and Houk, 1984). Polyspermy can occur when the

concentration of sperm lysin becomes so high that the vitelline membrane is completely dissolved, leading to low or no fertilisation (Lewis et al., 1982). Limiting lysin exposure thus reduces the likelihood of membrane destruction.

The optimal sperm density for fertilisation varies considerably between abalone; from 5 x 10^3 sperm ml^{-1} for *H. asinina* (Encena et al., 1998) to 10^6 sperm ml^{-1} for *H. laevigata* (Babcock and Keesing, 1999). This may arise through species differences in egg size, vitelline membrane permeability, lysin efficacy, success of the acrosome reaction or sperm morphology. While there are several accounts on the sperm morphology of haliotids (Lewis et al., 1980; Sakai et al., 1982; McCardle, 1984; Hodgson and Foster, 1992), including *H. laevigata* (Healy et al., 1998), these works do not discuss this factor in relation to observed differences in fertilisation success between species. An understanding of how sperm density, contact time and morphology interact to affect fertilisation success in blacklip and greenlip abalone is needed to produce more accurate models of the fertilisation biology of these species. Such models could aid in the formulation of management strategies by predicting a level of fishing that maintains adult density well above that where Allee effects occur.

This study of blacklip and greenlip abalone had three aims. Firstly, to document the combined effects of sperm density and contact time on the fertilisation rate of each species; secondly to compare these data between species, and finally to describe their sperm morphology (using scanning electron microscopy). These factors are discussed in relation to potential differences in fertilisation rate between species.

5.3 Methods

5.3.1 Spawning induction

Abalone were induced to spawn in individual 14 L tubs using heated, 1 µm-filtered, UV-irradiated seawater (Grubert and Ritar, 2002). Gametes were removed from male and female tubs within half an hour of initiation of spawning.

Eggs were siphoned into separate 2 L glass jars while sperm were poured into 20 L buckets and made up to a known volume. Egg suspensions were stirred and (1 ml) subsampled in triplicate to estimate egg density.

5.3.2 Quantification of sperm density

Sperm density was quantified spectrophotometrically. For each of three males of each species, sperm solution was transferred to a 25 ml cuvette and light absorbance (at 340 nm, following Kikuchi and Uki, 1974) determined using a Hach DR 2000 spectrophotometer (Hach Company, Loveland, Colorado). Triplicate readings were averaged to minimise the effects of air bubbles and shadowing by foreign matter. Following this, 2 ml of sperm was removed from the cuvette, placed in a watch glass and a drop of Lugol's iodine added to inactivate movement. After mixing, sperm counts were taken using a modified Neubauer's haemocytometer. The volume of sperm in the cuvette was reduced to 12.5 ml and the same volume of 1 μm-filtered seawater added to give a total volume of 25 ml. A series of absorbance readings, haemocytometer counts and two-fold dilutions were repeated until absorbance fell to the lowest detectable level (0.001).

5.3.3 Sperm-egg contact time and sperm density

Our experimental protocol generally followed that of Babcock and Keesing (1999). For each species, the effects of sperm-egg contact time and sperm density were assessed in a series of replicate experiments (2 males x 2 females for blacklips, 1 male x 2 females for greenlips) in which eggs were exposed to a range of sperm density x contact time combinations according to the following protocol. PVC tubes (20 mm in diameter) with one end covered by 63 μm mesh were placed in 200 ml glass jars containing sperm-free seawater and 1000 eggs pipetted into each tube. Tubes were simultaneously transferred to identical jars containing 100 ml of sperm at ca. 10^4, 10^5, 10^6 or 10^7 sperm ml^{-1}. After the contact time of 7, 15, 30, 60, 120, 240, 480, 1200 or 2400 s, eggs were removed from the sperm suspension, serially rinsed through six jars containing sperm-free seawater, and

transferred to 20 ml scintillation vials. Duplicate tubes were used for each male x female x sperm density x contact time combination. A positive control, in which eggs were exposed to an aliquot of stock solution (i.e. 1 x 10^7 for blacklips and 0.4 x 10^7 sperm ml^{-1} for greenlips – the latter being the highest sperm density available at the time) for 2 min, was run concurrently with each contact time treatment to determine if the viability of the stock solution changed over the course of the experiment. Negative controls were also run to detect possible contamination of sperm free water stocks. After each contact time trial, fresh sperm solutions were made up by adding 0.1, 1, 10 or 100 ml of the stock solution to 99.9, 99, 90 or 0 ml of sperm-free seawater, respectively. Embryos were incubated at room temperature (~22ºC) for 2 h then fixed in 2 ml of 10% formosaline. Visual assessment of fertilisation rate at 4-16 cell stage was conducted on 100 eggs from each replicate.

5.3.4 Preparation and examination of sperm using scanning electron microscopy

Sperm from three males of each species were centrifuged at 8000 x *g* for 15 min and the pellet fixed in 5% glutaraldehyde sucrose-phosphate buffer (pH 7.4) solution for 18 h at 4ºC. Samples were then transferred to 0.5 ml porous vessels and serially dehydrated in 70, 80, 90, 95 then 100 % methanol (20 min each), 100 % acetone (20 min) and hexamethyldisilazane (ProSciTech®; 2 × 30 min). Specimens were mounted on stubs, sputter coated with 21 nm of gold (Balzers SCD 004 sputter coater) and examined under high vacuum on a JEOL JSM-840 scanning electron microscope. Dimensions of ten sperm from each sample were estimated using SigmaScan Pro 5.0 software (SPSS Inc.).

5.3.5 Statistics

For intra-specific comparisons, data were arcsine transformed and two-way Analysis of Variance (ANOVA) used to test for significant differences in fertilisation rates between treatments (i.e. sperm-egg contact time and sperm density). One-way ANOVA and Tukey-Kramer HSD tests (for post-hoc

comparison) were then run to locate significant differences between contact time treatments at each of the four sperm densities. Kolmogorov-Smirnov and Bartlett's tests were used to check for normality and homogeneity of variance, respectively.

For inter-specific comparisons, data from each species and sperm density treatment were grouped by contact time and the proportion of eggs fertilised (P) transformed using the logit function (Finney 1978; Hewlett & Plackett 1979) where logit (P) = ln(P/1 – P). Linear regression of logit (P) values was then performed on log-transformed sperm density for each species x contact time treatment. In order to compare fertilisation success across a range of gamete contact times, the sperm density for 50% fertilisation (i.e. F_{50}) was derived from each regression by solving for logit(0.5) = 0. Analysis of Co-Variance (ANCOVA) was then performed on log-log transformed F_{50}-contact time data. Probabilities of <0.05 were considered significantly different. All statistics were executed using JMP 5.1 software (SAS Institute Inc.).

5.4 Results

5.4.1 Relationship between sperm density and light absorbance

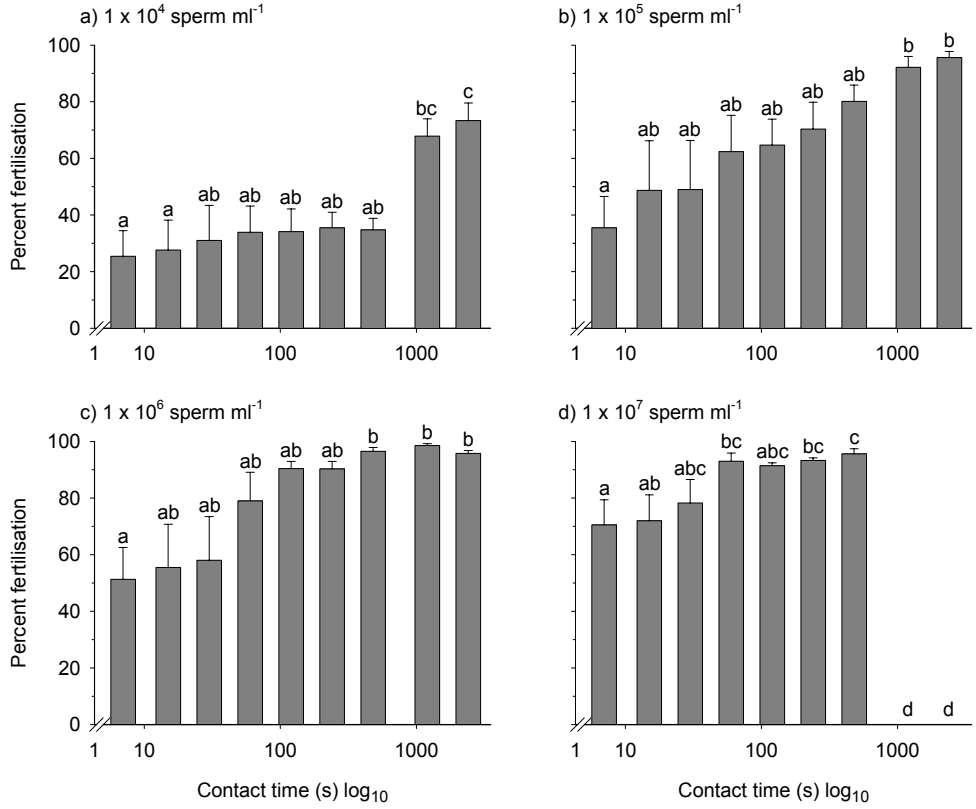
The relationship between sperm density (SD) and light absorbance at 340 nm (A) was $SD = 3^{7A^2} + 2^{7A}$; $r^2 = 0.99$ for blacklip abalone and $SD = 2^{7A^2} + 2^{7A}$; $r^2 = 0.98$ for greenlip abalone. These functions were used to estimate sperm density from absorbance in subsequent fertilisation trials.

5.4.2 The effect of sperm-egg contact time and sperm density on fertilisation of blacklip abalone (H. rubra)

There was a significant interaction between sperm density and contact time on the fertilisation success of blacklip eggs (Two-way ANOVA; df = 24, F = 7.79, $p < 0.001$). Short sperm-egg contact times (i.e. 7–480 s) at the lowest sperm density (1×10^4 sperm ml^{-1}) resulted in ≤ 35% fertilisation (Fig. 5.1a). Fertilisation rate almost doubled to 68% when the contact time was extended to

1200 s, but showed no appreciable change when this interval was doubled to 2400 s. When sperm density was increased to 1 x 10^5 sperm ml^{-1}, fertilisation rate increased proportionally with contact time, ranging from 36–96% (Fig. 5.1b). At 1 x 10^6 sperm ml^{-1}, fertilisation success was 51% after 7 s contact and increased to (and remained at) ≥ 95% by 480 s and thereafter (Fig. 5.1c). At the highest sperm density of 1 x 10^7 sperm ml^{-1}, fertilisation was 72% after 7 s contact and rose to ≥ 90% by 60 s, where it remained in all but the 1200 and 2400 s treatments (where only empty egg membranes remained; Fig 5.1d). Mean (± S.E.) fertilisation rates of positive controls for each contact time treatment were not significantly different (95 ± 2%), indicating that viability of the stock solution did not change over the course of the experiment. There was no fertilisation in the negative (sperm-free) controls.

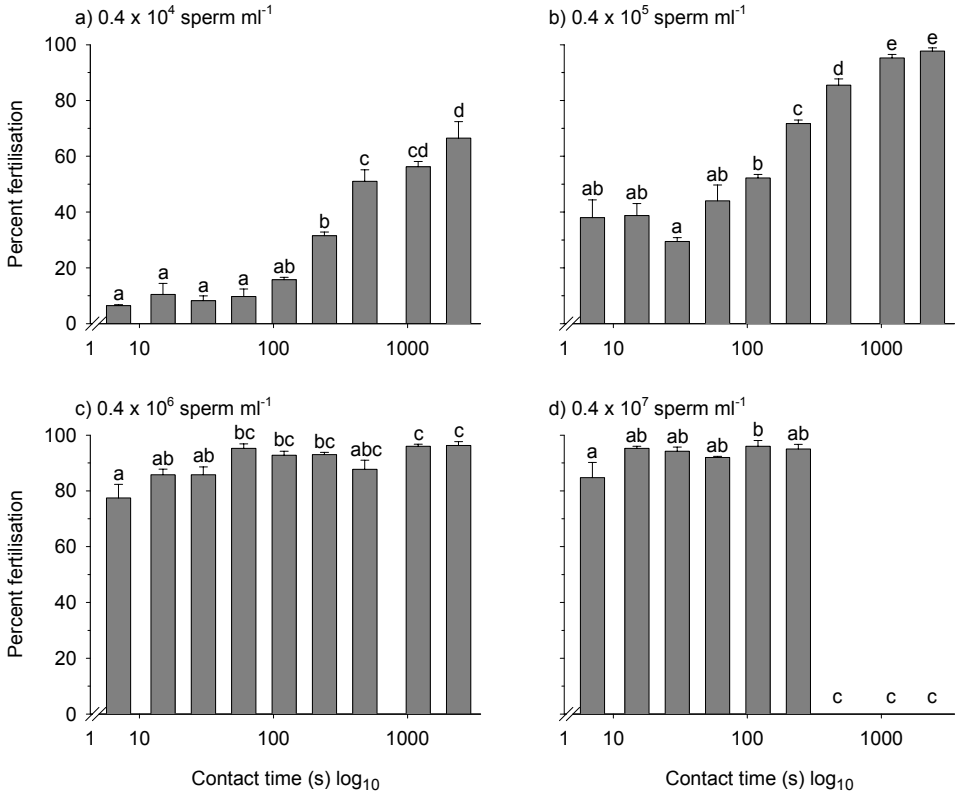
Figure 5.1 The effect of sperm-egg contact time at sperm densities of a) 1 x 10^4 sperm ml^{-1}, b) 1 x 10^5 sperm ml^{-1}, c) 1 x 10^6 sperm ml^{-1} and d) 1 x 10^7 sperm ml^{-1} on fertilisation success of *H. rubra*. Bars with the same letters within each density treatment are not significantly different.



5.4.3 The effect of sperm-egg contact time and sperm density on fertilisation of greenlip abalone (H. laevigata)

Similar to blacklip abalone, there was also a significant interaction between sperm density and contact time on the fertilisation success of greenlip eggs (Two-way ANOVA; df = 24, F = 88.22, $p < 0.001$). Short sperm-egg contact times (i.e. 7–60 s) at the lowest sperm density (0.4 x 10^4 sperm ml^{-1}) resulted in low (< 10%) fertilisation success (Fig. 5.2a). When contact time was increased beyond one minute, fertilisation rate rose proportionally, reaching 63% at 2400 s. An increase in sperm density to 0.4 x 10^5 sperm ml^{-1}, saw the fertilisation rate rise by 20–30% across all contact times and reach > 95% by 1200 s (Fig. 5.2b). At the two highest

Figure 5.2 The effect of sperm-egg contact time at sperm densities of a) 0.4 x 10^4 sperm ml^{-1}, b) 0.4 x 10^5 sperm ml^{-1}, c) 0.4 x 10^6 sperm ml^{-1} and d) 0.4 x 10^7 sperm ml^{-1} on fertilisation success of *H. laevigata*. Bars with the same letters within each density treatment are not significantly different.



sperm densities (i.e. 0.4 x 10^6 and 0.4 x 10^7 sperm ml^{-1}), fertilisation rates were > 75% for all treatments (Figs. 5.2c and 5.2d), the exceptions being the three longest exposure times of 480, 1200 and 2400 s at 0.4 x 10^7 sperm ml^{-1}, where fertilisation was nil. Again, there was no significant difference in the fertilisation rate of positive controls (96 ± 3%) indicating that the viability of the stock solution remained the same throughout the experiment. Likewise, there was no fertilisation in the negative (sperm-free) controls.

5.4.4 Comparison of fertilisation success between species

In blacklip abalone, the correlation coefficients for the relationship between logit (*P*) and $\log_{10}$sperm density increased proportionally with gamete contact time whereas in greenlip abalone the r^2 values remained high and varied little (Table 5.1). However, these patterns broke down at longer contact times (i.e. ≥ 1200 s in blacklip abalone and ≥ 480 s in greenlip abalone) due to a reduction in the data set (i.e. zero scores from polyspermic trials were omitted). Plots of logit (*P*) for each pair-wise cross vs $\log_{10}$sperm density for three contact times (i.e. 15, 60 and 240 s) show that between-cross variation was much greater than within-cross variation and that differences in fertilisation potential of particular sperm-egg combinations

Table 5.1 Slope (a), intercept (b), correlation coefficient (r^2) and F_{50} values for the relationship between Logit (*P*) and $\log_{10}$sperm density (sperm ml^{-1}) at different time intervals (s) for blacklip (BL) and greenlip (GL) abalone.

	BL				GL			
Time	a	b	r^2	F_{50}	a	b	r^2	F_{50}
7	0.82	4.67	0.24	$10^{5.70}$	1.57	7.99	0.88	$10^{5.09}$
15	0.88	4.23	0.12	$10^{4.81}$	1.88	9.10	0.92	$10^{4.84}$
30	1.16	5.88	0.20	$10^{5.07}$	1.89	9.29	0.94	$10^{4.92}$
60	1.40	6.39	0.43	$10^{4.56}$	1.79	8.35	0.76	$10^{4.66}$
120	1.13	4.95	0.55	$10^{4.38}$	1.82	8.14	0.92	$10^{4.47}$
240	1.16	4.96	0.61	$10^{4.28}$	1.36	5.47	0.89	$10^{4.02}$
480	1.46	6.00	0.70	$10^{4.11}$	1.02	3.38	0.70	$10^{3.31}$
1200	1.71	5.77	0.65	$10^{3.37}$	1.49	4.67	0.73	$10^{3.13}$
2400	1.06	2.57	0.36	$10^{2.42}$	1.38	3.66	0.52	$10^{2.65}$

decreased with increasing contact time (Fig. 5.3a-f). These trends were more obvious in blacklip abalone data than greenlip abalone data as only one male was used for the latter crosses. ANCOVA on log-log F_{50}-gamete contact time data revealed that contact time had a significant effect ($p < 0.0001$) whereas as species did not ($p = 0.22$). These data are visually represented in Fig 5.4.

Figure 5.3 Examples of the relationships between Logit (*P*) and Log_{10}sperm density (sperm ml^{-1}) for three gamete contact times for blacklip (BL) and greenlip (GL) abalone. Explanation of pair-wise crosses: filled symbols = female 1, open symbols = female 2; circles = male 1, squares = male 2.

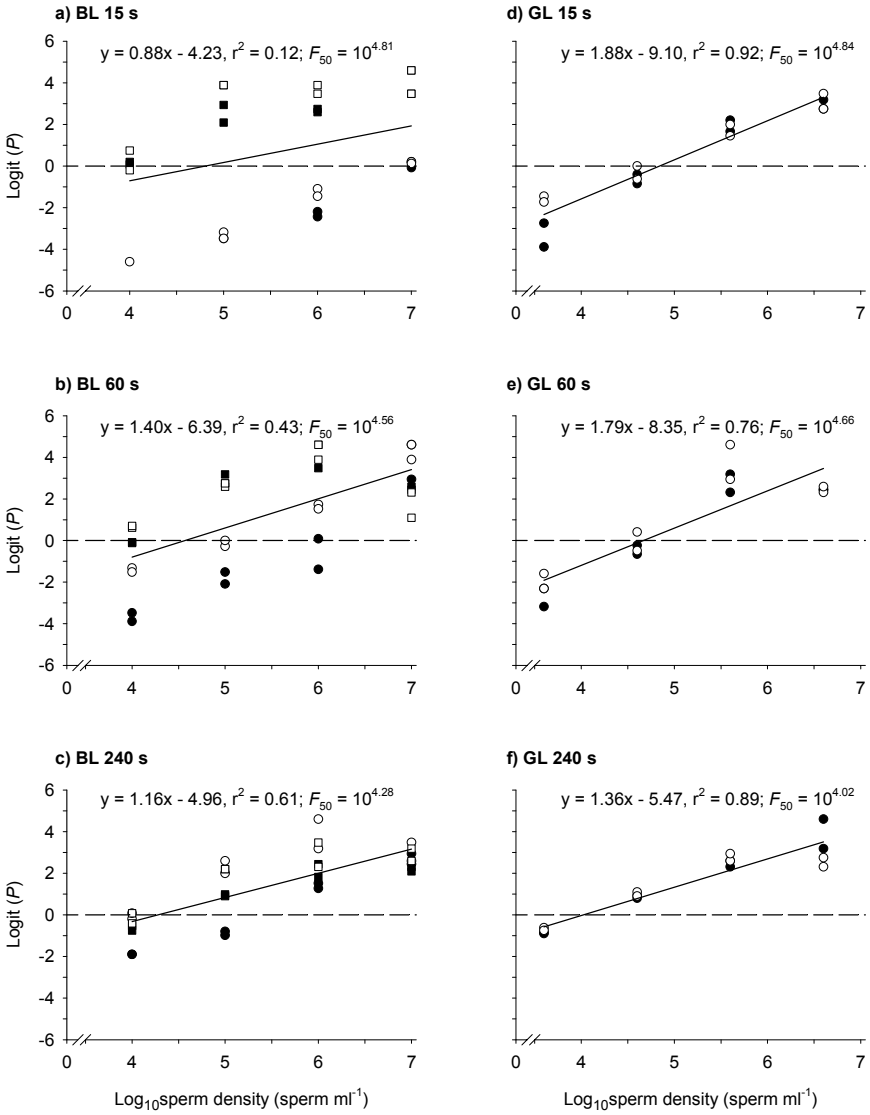
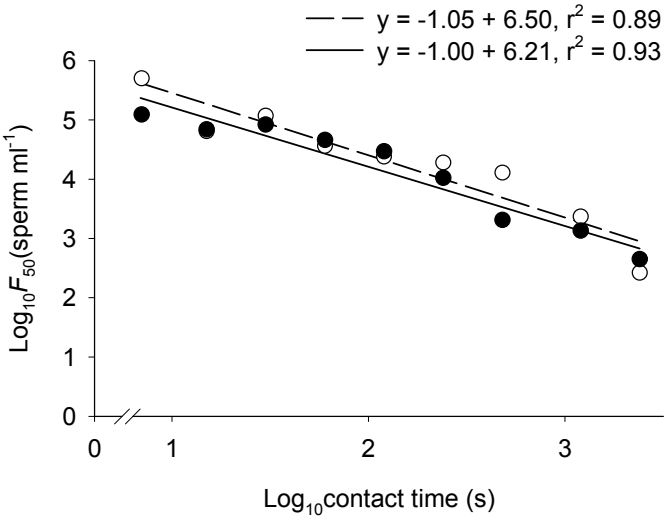


Figure 5.4 Linear regressions of $\log_{10}F_{50}$ vs $\log_{10}$contact time for blacklip (open circles, dashed line) and greenlip (filled circles, solid line) abalone.



5.4.5 Sperm morphology of blacklip (H. rubra) and greenlip (H. laevigata) abalone

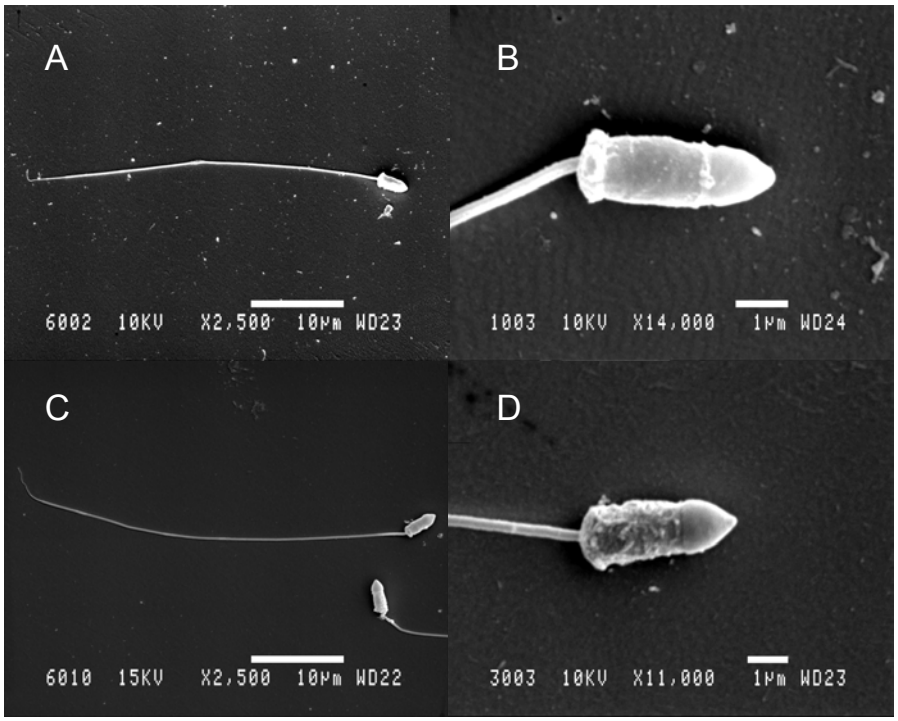
Electron micrographs of whole sperm and sperm heads from blacklip and greenlip abalone are shown in Fig. 5.5. The total length of blacklip abalone sperm (n = 10) was approximately 42.0 µm, consisting of a 1.3 µm acrosome, 2.1 µm nucleus, 0.6 µm midpiece (collectively known as the head) and 38.0 µm flagellum (Table 5.2). Corresponding lengths for greenlip abalone sperm (n = 10) were 48.8 µm, 1.3 µm, 2.2 µm, 0.3 µm and 45.0 µm, respectively. While the length of the acrosome was identical in both species, shape was slightly different, blacklips having a blunt tip and greenlips a V-shaped tip.

5.5 Discussion

This study showed that the fertilisation rate of *H. rubra* and *H. laevigata* eggs increased proportionally with gamete contact time at sperm densities between ca. 10^4 and 10^7 ml^{-1}, except for long contact periods (i.e. ≥ 1200 s for blacklip abalone and ≥ 480 s for greenlip abalone) at the highest density where eggs were destroyed, presumably by polyspermy. Furthermore, a comparison of F_{50} values

between species across a range of gamete contact times suggests that the fertilisation potential of blacklip and greenlip eggs is similar, at least within the range of treatments tested.

Figure 5.5 Scanning electron micrographs of (A–B) blacklip and (C–D) greenlip sperm



Our data exhibit different trends to those previously documented for blacklip (Huchette et al. 2004) and greenlip (Babcock & Keesing, 1999) abalone. Both these works recorded higher fertilisation rates at sperm densities of ca. 10^4 and 10^5 sperm ml^{-1} and lower fertilisation rates at ca. 10^6 and 10^7 sperm ml^{-1} (due to polyspermy) than those recorded here. This variation may be a consequence of differential egg size between studies, as Huchette et al. (2004) showed that 50% fertilisation of small eggs requires an order of magnitude greater sperm density than that of larger eggs. Furthermore, unintentional selection of small eggs (we did not measure egg size) from both *H. rubra* and *H. laevigata* in this study may explain why we derived comparable F_{50} values for both species. Whilst these findings require further investigation, this work and observed similarities in egg and sperm size ranges (Plant 2002; Huchette et al. 2004; this study) lend support to the idea that blacklip and greenlip show similar fertilisation responses.

Table 5.1 Comparison of dimensions of sperm components in selected Haliotidae. Dash indicates data not available.

Species	Sperm component length x diameter (µm)				Total Length	Source
	Acrosome	Nucleus	Midpiece	Flagellum		
H. australis	1.3 x 0.9	2.1 x 0.9	0.4 x 1.1	31 x 0.2	34.8	McCardle, 1984
H. discus	2.8 x 1.1	2.8 x 1.0	0.9 x 1.1	40 x –	46.5	Sakai et al., 1982
H. diversicolor[a]	1.0 x 1.3	1.5 x 1.3	0.6 x 1.1[b]	–	–	Shiroya and Sakai, 1992
H. diversicolor[c]	1.0 x 1.1	1.7 x 1.3	0.5 x 1.2[d]	–	–	Gwo et al., 1997
H. iris	1.6 x 1.4	1.4 x 1.2	0.6 x 1.2	38 x 0.2	41.6	McCardle, 1984
H. laevigata	1.4 x 1.4	2.2 x 1.5	0.8 x 1.7[e]	42 x 0.3	46.0	Healy et al., 1998[f]
	1.3 x 1.3	2.2 x 1.3	0.3 x 1.7	45 x 0.3	48.8	Present study
H. midae	3.1 x 0.9	2.8 x 1.0	–	–	–	Hodgson and Foster, 1992
H. rubra	1.3 x 1.0	2.1 x 1.0	0.6 x 1.6	38 x 0.3	42.0	Present study
H. rufescens	2.5 x 1.2	4.2 x 1.0	0.8 x 1.3	45 x 0.3	52.5	Lewis et al., 1980
H. virginea morioria	1.3 x 1.1	1.9 x 1.3	0.4 x 1.2	29 x 0.2	32.6	McCardle, 1984
H. virginea virginea	1.2 x 1.2	1.9 x 1.4	0.5 x 1.3	33 x 0.2	36.6	McCardle, 1984

a) as *Suculus diversicolor aquatilis*, see Geiger (1998) for a taxonomic review.
b) estimated from Shiroya and Sakai, 1992.
c) as *Suculus diversicolor supertexta*.
d) estimated from Gwo et al., 1997.
e) estimated from Healy et al., 1998.
f) values represent midpoint of range.

Although significant emphasis has been placed on the importance of sperm density and sperm-egg contact time on fertilisation of haliotids, sperm release rates are rarely documented (Uki and Kikuchi, 1982; Clavier, 1992; Babcock and Keesing, 1999). Unless males are able to release concentrated sperm (e.g. 10^7 ml^{-1}) frequently, then it cannot be assumed that sperm density effects observed in the laboratory (such as polyspermy) take place *in-situ*. Babcock and Keesing (1999) reported high sperm release rates (5.3 x 10^7 sperm s^{-1}) for

greenlips but stressed that high sperm densities would only be short lived due to dilution by water movement. Hence, synchronous spawning of several males in still water may be the only situation under which polyspermy would occur in nature. The current lack of direct observations of spawning in the field means there are insufficient data to either support or reject this hypothesis.

As per most other works on the fertilisation biology of abalone, our trials were conducted in static water. Thus, the trends we observed may not necessarily reflect those which occur in moving water. The eggs of at least one abalone (*H. rufescens*) release a sperm attractant (L-tryptophan), which under still conditions allow sperm to orientate and accelerate towards the egg (Riffel et al., 2002). This effectively doubles the target size of the egg, increasing the chance of gamete contact and fertilisation (Riffel et al., 2004). Clearly, the effectiveness of the sperm attractant will be inversely proportional to water movement and in cases where abalone spawn during (or shortly after) storms or typhoons (e.g. *H. discus hannai*, Sasaki and Shepherd, 1995) the effect of the attractant would be negligible. Under these circumstances, water movement rather than sperm chemotaxis dictates the probability of gamete contact. Thus, it appears that abalone have both direct and indirect mechanisms of maximising gamete contact under a variety of water conditions.

Both total and component length of abalone sperm differ between species, with blacklip and greenlip abalone sperm being of intermediate size (see Table 5.2). While species with a larger acrosome (presumably containing more sperm lysin) and/or a longer flagellum (perhaps conferring a greater swimming ability) could be expected to have a lower optimal sperm density for fertilisation, this does not appear to be the case. For example, both the acrosome and flagellum of blacklip sperm are smaller than that of *H. rufescens* sperm, yet the optimal sperm density for fertilisation of these species is similar (i.e. 1×10^6 sperm ml^{-1}; Mill and McCormick, 1992; this study).

One difference in the fertilisation dynamics of *H. rubra* and *H. laevigata* was the fact that the former was less prone to polyspermy at the highest sperm density. This may be due to differences in acrosomal morphology, the V-shaped head of greenlip sperm perhaps being more efficient at penetrating the vitelline membrane (particularly when it is softened by the high concentrations of sperm lysin) than the blunt head of blacklip sperm.

In conclusion, fertilisation success of blacklip and greenlip abalone is influenced by both sperm density and sperm-egg contact time. The combination of high sperm density (i.e. 10^7 ml^{-1}) and short ($\leq$ 30 s) contact times had no detrimental effect on fertilization success of either species, but prolonged exposure (i.e. $\geq$ 20 min) to concentrated sperm resulted in egg degradation, presumably because of polyspermy. The fertilisation potential of eggs from both species appeared similar, at least within the range of treatments used. However, given the limited data set, further work is required to clarify this matter. Future works of this nature should take into account gamete age, size, shape, concentration and contact time. For studies in the wild, the role of chemoattractants, adult density, synchronicity of spawning and water movement should also be examined.

5.6 Acknowledgements

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Chapter 6 The effect of temperature on the embryonic and larval development of blacklip (*Haliotis rubra*) and greenlip (*H. laevigata*) abalone

6.1 Abstract

Embryonic and larval development of *Haliotis rubra* and *H. laevigata* was observed at water temperatures of 12–20ºC from fertilisation to completion of the velum. The timing of first and second cell division, prototrochal cilia formation and completion of the velum was plotted against water temperature to determine the Biological Zero Point (i.e. the theoretical minimum temperature below which larval development is arrested) for each species. The BZP estimate for larval development of *H. rubra* was 7.8ºC and of *H. laevigata* was 7.2ºC. A simultaneous experiment, in which larvae were cultured at ca. 16ºC and sampled at 4 h intervals determined the Effective Accumulative Temperature (EAT; the cumulative difference between the culture temperature and the BZP, calculated hourly) for prominent developmental stages in both species. The EAT for hatchout, torsion, eyespot formation and metamorphic competence (i.e. formation of the fourth tubule on the cephalic tentacle) for blacklip abalone was 160, 380, 590 and 1280 EATºC-h, respectively. Corresponding figures for greenlip abalone were 180, 420, 640 and 1340 EATºC-h, respectively. The EAT for dispersal (i.e. the difference between the EAT for metamorphic competence and that for hatchout) was 1120 and 1160 EATºC-h for blacklip and greenlip abalone, respectively. Such information enables the prediction of the dispersal window for each species at a given temperature.

6.2 Introduction

The effect of temperature on gonad development of blacklip (*Haliotis rubra*) and greenlip (*H. laevigata*) abalone is well documented (Lleonart, 1992; Grubert and Ritar, 2004). By contrast, its effect on embryonic and larval development is restricted to the works of Harrison and Grant (1971) and Grant and Sumner (unpublished) on *H. rubra*. As yet, no studies have documented the early development of either species across their normal thermal range (8–22ºC for blacklip abalone and 12–22ºC for greenlip abalone; Gilroy and Edwards, 1998; Hone and Fleming, 1998).

The early life history of abalone includes two motile, planktonic, lecithotrophic (i.e. non-feeding) stages; the trochophore and the veliger. The length of the trochophore stage is brief (a few hours), while that of the veliger stage is much longer (days/weeks). The duration of these stages is temperature dependant, which in turn affects the ability of the larvae to disperse. Higher temperatures result in more rapid development, reduced dispersal time, and less exposure to predators, while at lower temperatures the situation is reversed. Hence, temperature has both direct and indirect effects on the early life history of abalone.

Several works have shown that larval development of abalone is arrested below a certain threshold temperature or "biological zero point" (BZP). The BZP for larval development varies from 5–9ºC for temperate abalone (Seki and Kan-no, 1977; Kabir, 2001) and 10–15ºC for tropical species (Bang and Han, 1993; Sawatpeera et al., 2001). At temperatures above the BZP, the appearance of each developmental stage is a function of the cumulative difference (calculated hourly) between culture temperature and the BZP. The value of this function, known as the Effective Accumulative Temperature (EAT; expressed in ºC-h) is constant for each stage and once quantified, provides a means of predicting the onset of each stage at temperatures below the species' upper thermal limit. This enables the duration of the "dispersal window" (i.e. the time between hatchout and metamorphic competence) for abalone larvae to be calculated from water

temperature (assuming that metamorphosis is not further delayed through the absence of suitable induction agents). The aim of this study was to determine the BZP and EAT for larval development of blacklip and greenlip abalone. This information can then be used in models of larval transport for these species.

6.3 Methods

6.3.1 Spawning induction

Wild-caught blacklip and greenlip abalone broodstock were induced to spawn using heated, UV-irradiated seawater (Grubert and Ritar, 2002). Gametes were collected from one animal of each sex and quantified within an hour of spawning. Sperm density was estimated using a spectrophotometer (at 340 nm, Ritar and Grubert, 2002), and egg counts made on triplicate samples diluted to 10:1 from the original solution. Sperm were diluted to 10^7 sperm ml^{-1} (the stock solution) using UV-treated, 1 µm filtered seawater (UV-FSW).

6.3.2 Experiment 1: Early development and Biological Zero Point (BZP) estimation

The culture system consisted of 70 ml polystyrene jars maintained at ca. 12ºC, 14ºC, 16ºC, 18ºC and 20ºC in an aluminium temperature gradient block. Prior to insemination, eggs and sperm were placed in separate jars at each temperature and allowed to equilibrate for 10 min. Fifty ml of stock sperm solution was added directly to each "male" jar. Approximately 10^4 eggs were added to "fertilisation sieves" (20 mm diameter PVC tubes with a 63 µm mesh base) immersed in 20 ml of UV-FSW inside each "female" jar. Following equilibration, the sieves were transferred to the sperm solutions for 3 min then serially rinsed through three jars containing UV-FSW at the same temperature. Eggs were then washed into empty jars with 50 ml of isothermal UV-FSW. At 6 min post insemination (PI), 0.5 ml of egg suspension was removed from each temperature treatment and transferred to numbered 2 ml microfuge tubes containing 0.5 ml of 10% formosaline. Further

samples were collected every 2 min until 2 h PI then every 5 min until 4 h PI. Thereafter, direct observations (under a Nikon SMZ-1 dissecting microscope) were made every 4 h until 40 h PI, at which time the trial ceased. Fixed samples were examined under a Nikon Optiphot phase contrast microscope.

For each species, the timing of 1st and 2nd cell division, prototrochal cilia formation, hatchout and completion of the velum was used to estimate the BZP. This was achieved by calculating the reciprocal of the time taken for at least 50% appearance of each stage at each temperature then plotting these values against temperature. The x-intercept for each linear regression line was then extrapolated (using inverse prediction; Zar, 1996) and these values averaged to yield the BZP.

6.3.3 Experiment 2: Effective Accumulative Temperature (EAT) for larval development

Eggs were siphoned into a 63 µm sieve, immersed in a fresh sperm solution (10^7 sperm ml^{-1}) for 3 min, serially rinsed through three 20 L buckets containing 5 L UV-FSW then washed into another 20 L bucket. Eggs were evenly dispersed by stirring and equal volumes transferred to each of 10 downwellers. The downwellers (90 mm diameter PVC tubes, 16 cm high, with a 63 µm mesh screen at the base, ~600 ml effective volume) were arranged inside a polypropylene basin (80 x 52 x 20 cm, L x W x D) and UV-FSW at ~16ºC supplied to each at a rate of approximately 100 ml min^{-1}. Each downweller was sampled every 2 h from 6–24 h PI then at 4 h intervals until the larvae attained metamorphic competence (i.e. formation of the fourth tubule on the cephalic tentacle). Observations of larval development were made under a Nikon SMZ-1 dissecting microscope. The EAT for each stage was calculated by subtracting the BZP from the culture temperature then multiplying by the number of hours to reach the stage.

6.4 Results

6.4.1 Experiment 1: Early development and Biological Zero Point (BZP) estimation

The interval between insemination and polar body 1 (PB1) and polar body 2 (PB2) release decreased with increasing culture temperature in both species (Table 6.1). In blacklip embryos, PB1 extrusion commenced at 6–10 min PI and peaked at 16–46 min PI, while extrusion of PB2 began at 14–30 min PI and peaked at 38–110 min PI. Corresponding figures for greenlip embryos were 6–8 min and 14–36 min PI for PB1 and 12–28 min and 30–74 min PI for PB2. Similarly, 1st and 2nd cell division, prototrochal cilia formation, hatchout and completion of the velum, occurred earlier as temperature increased, and the relationships are described for blacklip and greenlip abalone in Figs. 6.1a and b, respectively. The interpolation of these relationships to the x-axis gave mean (± s.e.) larval BZP estimates of 7.8 ± 0.0ºC and 7.2 ± 0.1ºC for blacklip and greenlip abalone, respectively (Table 6.2).

Table 6.1 Observed start ($Time_{start}$) and peak ($Time_{peak}$) release times (minutes post insemination) of polar bodies 1 and 2 (PB1 and PB2, respectively) for blacklip (BL) and greenlip (GL) embryos held at different temperatures (Temp.).

		PB1		PB2	
Species	Temp. (ºC)	$Time_{start}$	$Time_{peak}$	$Time_{start}$	$Time_{peak}$
BL	12.6	10	46	30	110
	14.5	8	30	26	78
	16.3	8	24	18	54
	18.1	6	18	16	46
	20.4	6	16	14	38
GL	12.4	8	36	28	74
	14.4	8	24	24	52
	16.2	8	20	18	42
	18.1	6	16	16	34
	20.4	6	14	12	30

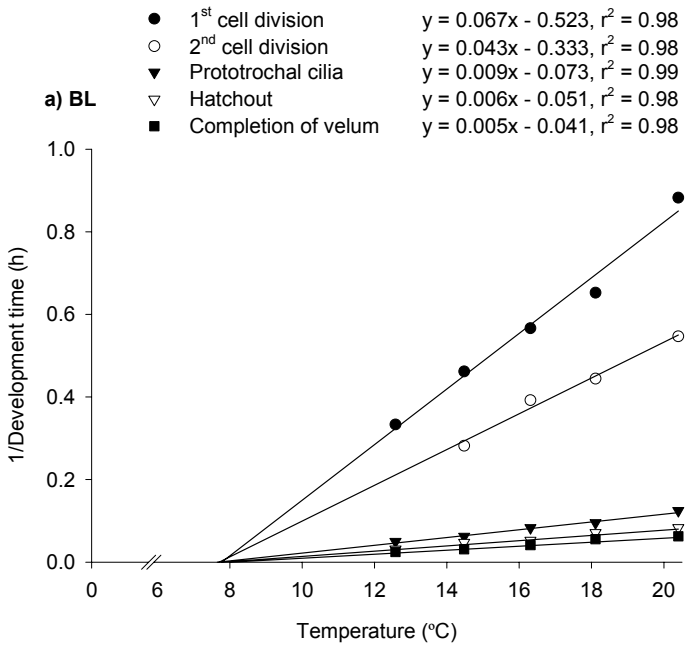
Table 6.2 Upper and lower 95% confidence intervals (CI) for BZP estimates (in ºC) of selected embryonic and larval stages of blacklip and greenlip abalone.

Stage	Blacklip			Greenlip		
	BZP	Upper 95% CI	Lower 95% CI	BZP	Upper 95% CI	Lower 95% CI
First cell division	7.8	9.6	4.8	7.0	7.6	6.8
Second cell division	7.8	10.6	5.1	7.4	8.3	5.9
Prototrochal cilia	7.7	10.1	5.4	7.2	9.8	5.3
Hatchout	7.8	10.3	5.2	6.7	8.0	4.9
Completion of velum	8.0	11.1	4.2	7.5	9.4	4.6
Average BZP (± S.E)	7.8 ± 0.0			7.2 ± 0.1		

6.4.2 Experiment 2: Effective Accumulative Temperature (EAT) for larval development

The timing of hatchout, torsion, eyespot formation and metamorphic competence (i.e. formation of the fourth tubule on the cephalic tentacle) for blacklip larvae cultured at 16.9ºC was 18, 42, 66 and 142 h PI, respectively (Table 6.3). Using a BZP of 7.8ºC, this equated to 160, 380, 590 and 1280 EATºC-h, respectively. Corresponding times for greenlip larvae cultured at 16.4ºC were 20, 46, 70 and 146 h PI and (using a BZP of 7.2ºC) 180, 420, 640 and 1340 EATºC-h, respectively. The EAT for dispersal (i.e. the difference between the EAT for metamorphic competence and that for hatchout) was 1120 and 1160 EATºC-h for blacklip and greenlip abalone, respectively.

Figure 6.1 The relationship between the reciprocal of development time and temperature for selected embryonic and larval stages of (a) blacklip (BL) and (b) greenlip (GL) abalone.



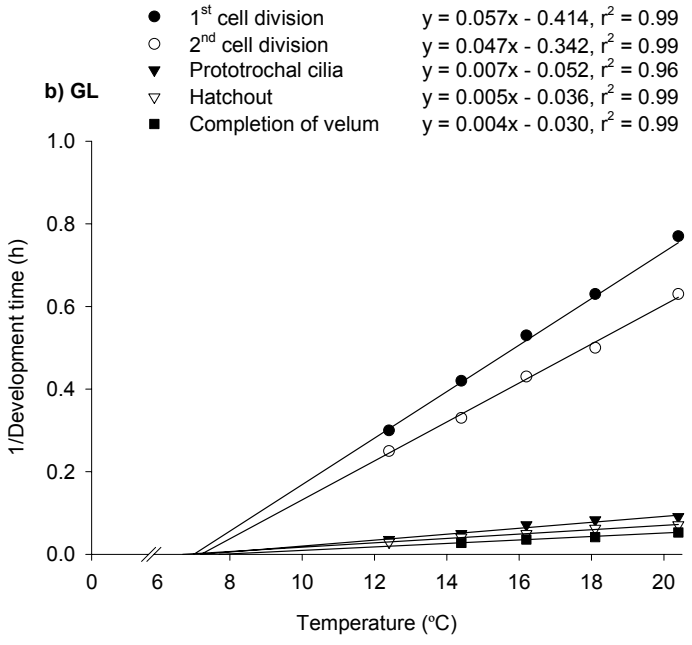


Table 6.3 Interval from insemination to the appearance of embryonic and larval stages (in hours and effective accumulative temperature – EATºC-h) for blacklip and greenlip abalone held at 16.9ºC and 16.4ºC, respectively. *Other stages were not characterised by the 4 h sampling regime.

	Blacklip (16.9ºC)		Greenlip (16.4ºC)	
Stage*	Time (h)	EATºC-h	Time (h)	EATºC-h
Prototrochal cilia	10	90	14	130
Hatchout	18	160	20	180
Completion of velum	22	200	28	260
Integumental attachment	38	340	42	390
Torsion	42	380	46	420
Operculum	46	410	50	460
Groove in velum	54	490	58	530
Cilia on foot	58	520	62	570
Eyespot	66	590	70	640
Formation of propodium	74	670	78	720
Cilia on propodium	78	700	82	750
Cilia in mantle cavity	82	740	86	790
Cephalic tentacle	90	810	94	860
Apophysis on propodium	94	850	98	900
1st tubule formation	114	1030	118	1090
Statolith	118	1060	122	1120
2nd tubule formation	122	1100	130	1200
Ciliary process in mantle cavity	130	1170	134	1230
3rd tubule formation	134	1210	142	1310
4th tubule formation	142	1280	146	1340

6.5 Discussion

As is the case for several other abalone species (e.g. *H. discus hannai* and *H. australis*, Kikuchi and Uki, 1974; Seki and Kan-no, 1977; Kabir, 2001), larval BZP estimates for *H. rubra* and *H. laevigata* (7.8ºC and 7.2ºC, respectively) were similar to the BZP estimates for gonad development (7.8ºC and 6.9ºC for blacklip and greenlip abalone, respectively; Grubert and Ritar, 2004). Hence, temperature has a profound influence on both the gonadal and larval development in these species.

Comparison of BZP and EAT values for larval development of temperate and tropical abalone demonstrates two points. Firstly, BZP estimates for temperate species (e.g. *H. rubra* and *H. laevigata*) are less than those for tropical/subtropical

species (e.g. *H. asinina* and *H. diversicolor*; Table 6.4). Secondly, the EAT for metamorphic competence (i.e. the formation of the fourth tubule on the cephalic tentacle) of tropical species (e.g. 680 EATºC-h in *H. asinina*; Sawatpeera et al., 2001) is much less than that for temperate species (e.g. 1340 EATºC-h in *H. laevigata*; this study). Therefore, the following discussion is restricted to comparisons of larval development of temperate abalone.

Table 6.4 Larval biological zero point (BZP) estimates and effective accumulative temperature (EAT) for hatchout and metamorphic competence (MC) of selected Haliotidae.

Species	Larval BZP (ºC)	Hatchout (EATºC-h)	MC (EATºC-h)	Source
H. asinina	15.0	100	680	Sawatpeera, *et al*., 2001
H. australis	5.0	225	–	Kabir, 2001
H. corrugata	5.7[a]	175[b]	1970[b]	Seki and Kan-no, 1977
H. discus discus	8.5	155	–	Seki and Kan-no, 1977
H. discus hannai	7.6	170	1220	Seki and Kan-no, 1977
H. diversicolor[c]	10.6	120	–	Bang and Han, 1993
H. fulgens	9.9[a]	100[b]	2560[b]	Seki and Kan-no, 1977
H. gigantea	9.0	140	–	Seki and Kan-no, 1977
H. laevigata	7.2	180	1340	This study
H. rubra	7.8	160	1280	This study
H. rufescens	8.5[a]	205[b]	–	Seki and Kan-no, 1977

a) calculated by Seki and Kan-no (1977) using data from Leighton (1974).

b) calculated using data from Leighton (1974).

c) as *Suculus diversicolor aquatilis*, see Geiger (1998) for taxonomic review.

– indicates data not available

The EAT values for hatchout of *H. rubra* (160 EATºC-h) and *H. laevigata* (180 EATºC-h) were similar and approximated the corresponding value for *H. discus hannai* (170 EATºC-h; Seki and Kan-no, 1977). All three intervals are considerably shorter than that for the New Zealand species *H. australis* (225 EATºC-h; Kabir, 2001). Torsion occurred at 380 EATºC-h and 420 EATºC-h in the larvae of blacklip and greenlip abalone, respectively. Again, these intervals are similar to that for *H. discus hannai* (390 EATºC-h), but greater than those for *H. discus discus* and *H. gigantea* (330 EATºC-h in both species; Seki and Kan-no, 1977).

Metamorphic competence was attained after 1280 EATºC-h in blacklip larvae and 1340 EATºC-h in greenlip larvae. These intervals are slightly longer than that for *H. discus hannai* (1220 EATºC-h; Seki and Kan-no, 1977) but considerably shorter than those for *H. corrugata* and *H. fulgens* (1970 EATºC-h and 2560 EATºC-h, respectively; Leighton, 1974; Seki and Kan-no, 1977).

The length of the "dispersal window" (i.e. the difference between the EAT for metamorphic competence and that for hatchout) was 1120 and 1160 EATºC-h for blacklip and greenlip abalone, respectively. Using these figures, it is possible to predict the length of the dispersal window at a given temperature. At 12ºC, the pelagic stage of blacklip larvae is predicted at 11.1 days, whereas at 20ºC, the estimate is 3.8 days. Corresponding figures for greenlip abalone are 10 and 3.8 days, respectively. These figures represent minimum dispersal times, as some abalone (e.g. *H. iris*) can delay metamorphosis for up to two weeks, without compromising subsequent growth and survival, if no suitable induction cues are detected (Roberts and Lapworth, 2001). However, such delays in metamorphosis lead to reduced survival and growth of *H. rufescens* larvae (Searcy-Bernal, 1999 cited by Roberts and Lapworth, 2001). Hence, the effects of delayed metamorphosis on blacklip and greenlip larvae need to be quantified to determine the upper limit for the dispersal window of these species.

The concept of a dispersal window assumes that larvae conform to the diffusion model, entering the water column as trochophores, passively transported by water currents, and concentrated in eddies and stagnation zones (Sasaki and Shepherd, 1995). The alternative philopatric model (proposed by Prince et al., 1987) asserts that larvae remain benthic during the veliger stage and have a limited capacity for dispersal. Prince et al. (1987) questioned whether larval behaviour observed in the laboratory, such as positive phototaxis, was indicative of that in the natural environment. They implied that positive phototaxis may in fact be a laboratory artefact (presumably because of differences between artificial and natural light) and that in the wild, larvae do not necessarily swim towards the illuminated surface waters and so do not disperse. However, most of the works cited by Prince

et al. (1987) failed to discriminate between positive phototaxis and negative geotaxis, whereas a recent study by Madigan (2000) on blacklip and greenlip abalone showed that larvae of both species are negatively geotactic during the veliger stage. Clearly, Madigan's (2000) findings support the diffusion model of larval dispersal.

While temperature affects the duration of the pelagic stage, the distance traveled during this period is a function of water movement. Hence, if larvae develop in warm (e.g. 20ºC), still conditions, their potential to disperse will be much less than if they did so in cold (e.g. 12ºC), fast moving water. Therefore, models of larval transport for abalone must take into account the conditions under which each particular species spawns and the resultant larvae develop.

This study showed that the rate of larval development in both blacklip and greenlip abalone was dependent on the cumulative difference between the culture temperature and the BZP. The values for the BZP, hatchout and metamorphic competence can now be used to predict the minimum dispersal time for each species at a given temperature. Further work on the effects of delayed metamorphosis and larval transport (e.g. using fluorochrome tagged larvae and larval collectors) needs to be conducted to determine the upper limit of the dispersal window and extent of larval dispersal *in-situ*.

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Chapter 7 General Discussion

7.1 Factors influencing reproductive development

7.1.1 Gonadogenesis and spawning

This work showed that temperature affects both gonad development and spawning success in blacklip (*Haliotis rubra*) and greenlip (*H. laevigata*) abalone, and that both species conform to the BZP/EAT model of reproductive development (Chapters 2 and 3). Estimates of the BZP for gonad development of *H. rubra* and *H. laevigata* (7.8ºC and 6.9ºC, respectively) were within the range of values previously reported for temperate species (5–9ºC; Kikuchi and Uki, 1974a,b; Kabir, 2001) but estimates based on different indices (i.e. the VGI, MGBI and EV) varied, those derived from the rate of change in the MGBI (BZP_{MGBI}) being up to 2ºC lower than those from the VGI (BZP_{VGI}) or EV (BZP_{EV}). The BZP_{VGI} estimate was adopted in preference to the others as it was both easy to determine and attainable from either sex. These values can now be used to derive the time interval necessary to reach the optimal (EAT) conditioning interval for spawning (detailed below).

The means of deriving the estimate of gonad volume (EGV – which is divided by shucked wet weight to yield the MGBI) used here followed that of Lleonart (1992). His formula, based on area, rather than linear measurements of gonad cross-sections (c.f. Tutschulte and Connell, 1981 and Ault, 1984), is both simpler to compute and more accurate than previous formulae, as it is less prone to the distorting effects of formalin fixation.

An examination of oocyte microstructure during conditioning revealed that oocyte diameter ratios varied widely (Chapter 2), suggesting that the commonly used method of calculating oocyte volume in abalone (using spherical volume, $4/3.\pi.\{mean_r\}^3$) is inappropriate, as it leads to over estimates in all but perfectly round oocytes. An alternative (ellipsoid) volume formula ($4/3.\pi.max_r.\{min_r\}^2$)

was employed and was shown to provide a more accurate estimate. Hence, future studies of ovarian development in abalone (and possibly other species) should use the formula for an ellipsoid to calculate oocyte volume, particularly when oocyte diameter ratios are variable.

Oocyte size categories in tables of standardized residuals were presented in geometric, rather than arithmetic progression (c.f. Kabir, 2001) as the increase in oocyte volume is a cubic, rather than linear function. A convention whereby oocyte size is derived using ellipsoid volume and growth expressed in geometric progression (as done here), would aid in comparisons of oocyte development both within and between species.

A series of conditioning and spawning trials conducted over two consecutive cycles revealed that gamete production of blacklip and greenlip abalone was higher when conditioned at 16ºC rather than 18ºC (Chapter 3). The optimal conditioning interval (defined as the interval which yielded the highest spawning rate, repeat spawning rate and/or gamete production) for *H. rubra* was ≥1540 EATºC-days for males and ≥1350 EATºC-days for females (which equates to ≥188 and ≥165 days at 16ºC, respectively). Corresponding figures for *H. laevigata* were ≥1700 EATºC-days for males and ≥1930 EATºC-days for females (which equates to ≥188 and ≥212 days at 16ºC, respectively). These intervals fall within the range of values reported for Japanese and New Zealand abalone (1400–3500 EATºC-days; Kikuchi and Uki, 1974a,b; Kabir, 2001).

Using daily water temperature and the BZP and EAT values derived here, it is now possible to predict or deduce spawning events in both culture and natural environments (at least where food is not limiting). This information will benefit both fisheries biologists and hatchery managers as it will aid in the development of recruitment models and lead to more successful spawning inductions, respectively.

7.1.2 Somatic and gonadal tissue biochemistry

While a difference in conditioning temperature of 4ºC (i.e. 14ºC vs 18ºC) had a significant effect on the rate of gonad development in *H. rubra* and *H. laevigata*, it was insufficient to elicit a change in the lipid or FA composition of the foot, digestive gland (DG), testis or ovary in either species (Chapter 4). Likewise, these compositions did not differ between spent and gravid individuals. Hence, both species appear to be able to regulate their lipid and FA composition in response to different environmental temperatures (at least within the range of 14ºC–18ºC). Furthermore, the interval between spawning induction and sampling was too short to allow for resorption of residual gonad tissue (which may have been exacerbated by partial spawning), resulting in similar FA profiles for pre- and post-spawning animals.

Lipid levels in each tissue varied little both within and between species, with values ranging from 4–6%, 14–15%, 8–9% and 30–32 in the foot, DG, testis and ovary, respectively. The ranges are similar to those reported for captive abalone fed a formulated feed (Dunstan et al, 1996), but much narrower than those recorded for wild caught abalone (which are exposed to a more variable environment; Webber, 1970; Litaay and De Silva 2003).

Each tissue had a different FA signature, with the foot, testis and ovary characterized by elevated levels of 20:4n-6 (arachidonic acid – ARA), 20:5n-3 (eicosapentaenoic acid – EPA) and 18:2n-6 (linoleic acid – LNL), respectively. The proportion of LNL and EPA in the DG were intermediate between those of the testis and ovary.

Further work using a more intensive sampling regime is required to fully understand the lipid and FA dynamics during hatchery conditioning of blacklip and greenlip abalone. A wider range of experimental temperatures (e.g. 10 vs 22ºC) should also be trialed to determine the extent to which these species can regulate their lipid and FA composition in response to different environmental temperatures.

7.2 Factors influencing early life history

7.2.1 Fertilisation biology

There was a significant interaction between sperm density (10^4–10^7 sperm ml^{-1}) and gamete contact time (7–2400 s) on the fertilisation rate of both *H. rubra* and *H. laevigata* (Chapter 5). Prolonged exposure (i.e. 1200–2400 s for blacklips and 480–2400 s for greenlips) to concentrated sperm (i.e. 10^7 sperm ml^{-1}) resulted in lysis of the egg membrane and polyspermy. Furthermore, a comparison of F_{50} values between species across a range of gamete contact times suggests that the fertilisation potential of blacklip and greenlip eggs is similar, at least within the range of treatments tested. An examination of sperm morphology in each species revealed similarities in sperm length (i.e. 42–46 μm) and differences in the shape of acrosome, the tip of which was blunt in blacklip sperm and V-shaped in greenlip sperm. Slight differences in acrosomal morphology may explain why *H. laevigata* is more sensitive to higher sperm densities than *H. rubra*.

Whilst an understanding of the effects of sperm density and gamete contact time on fertilisation success of *H. rubra* and *H. laevigata* will assist in the development of models of the early life history of these species, much remains to be known of the role of other morphological (e.g. gamete size and shape), physiological (e.g. chemoattractants), behavioral (e.g. adult density and synchronicity of spawning) and environmental (e.g. water movement) factors on the fertilisation kinetics of these and other haliotid species.

7.2.2 Larval development

Estimates of the BZP for larval development of *H. rubra* and *H. laevigata* were 7.8ºC and 7.2ºC, respectively (Chapter 6). The EAT for hatchout, torsion, eyespot formation and metamorphic competence (i.e. formation of the fourth tubule on the cephalic tentacle) for blacklip abalone was 160, 380, 590 and 1280 EATºC-h, respectively. Corresponding figures for greenlip abalone were 180, 420, 640 and 1340 EATºC-h, respectively. The EAT for dispersal (i.e. the difference between

the EAT for metamorphic competence and hatchout) was 1120 and 1160 EATºC-h for blacklips and greenlips, respectively. This equates to a planktonic phase of 11.1 days for *H. rubra* and 10 days for *H. laevigata* at a water temperature of 12ºC. By contrast, the interval for both species is reduced to 3.8 days at 20ºC.

These estimates should be treated as minimum dispersal times as some abalone larvae can delay metamorphosis if no suitable induction cues are detected (Roberts and Lapworth, 2001). Hence, the ability of blacklip and greenlip abalone to delay metamorphosis and its effect on subsequent survival and growth needs to be investigated so as to determine the upper limit of the dispersal window for these species.

7.3 Guidelines for hatchery production of blacklip and greenlip abalone

From this work it is now possible to describe the optimal regimen for broodstock conditioning, fertilisation and larval rearing of *H. rubra* and *H. laevigata*. The values of these parameters are given in Table 7.1.

Table 7.1 Optimal broodstock conditioning, fertilisation and larval rearing regimes for blacklip and greenlip abalone.

Factor		Blacklip abalone	Greenlip abalone
Gonadal BZP		7.8ºC	6.9ºC
Conditioning temperature		16ºC	16ºC
Conditioning interval (EATºC-days)	M	≥ 1540	≥ 1700
	F	≥ 1350	≥ 1930
Conditioning interval (days at 16ºC)	M	≥ 188	≥ 188
	F	≥ 165	≥ 212
Sperm density (ml^{-1}) for fertilisation		10^6 for 4 min	10^6 for 4 min
Larval BZP		7.8ºC	7.2ºC
EAT for hatchout (ºC-h)		160	180
Time to hatchout (hrs at 16ºC*)		18	20
EAT for metamorphosis (ºC-h)		1280	1340
Time to metamorphosis (days at 16ºC*)		6.5	6.3

*suggested larval rearing temperature

7.4 Summary

This study demonstrated that temperature influences several aspects of the life history of blacklip and greenlip abalone. In both species, the rate of gonadal and larval development was proportional to the cumulative difference between water temperature and the BZP, while gamete production at spawning inductions was higher when cultured at 16ºC than 18ºC. Temperature did not however affect the lipid or FA composition of either species, at least within the range of 14ºC–18ºC. Fertilisation success of *H. rubra* and *H. laevigata* increased relative to sperm concentration and gamete contact time but high values for both factors led to lysis of the egg membrane and polyspermy.

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Appendix 1.

Analysis of CoVariance (Blacklip VGI at 12ºC)

Source	*df*	*SS*	*MS*	*F Ratio*	*Prob > F*
Model	2	13.50	6.75	31.07	<.0001
Error	40	8.69	0.22		
C. Total	42	22.19			

Effect test

Source	Nparm	*df*	*SS*	*F Ratio*	*Prob > F*
12 sex	1	1	0.05	0.25	0.62
12 time	1	1	13.17	60.61	<.0001

Analysis of CoVariance (Blacklip VGI at 14ºC)

Source	*df*	*SS*	*MS*	*F Ratio*	*Prob > F*
Model	2	17.00	8.50	45.49	<.0001
Error	51	9.53	0.19		
C. Total	53	26.54			

Effect test

Source	Nparm	*df*	*SS*	*F Ratio*	*Prob > F*
14 sex	1	1	0.05	0.28	0.60
14 time	1	1	16.57	88.67	<.0001

Analysis of CoVariance (Blacklip VGI at 16ºC)

Source	*df*	*SS*	*MS*	*F Ratio*	*Prob > F*
Model	2	55.45	27.72	115.91	<.0001
Error	72	17.22	0.24		
C. Total	74	72.67			

Effect test

Source	Nparm	*df*	*SS*	*F Ratio*	*Prob > F*
16 sex	1	1	0.00	0.01	0.93
16 time	1	1	55.44	231.81	<.0001

Analysis of CoVariance (Blacklip VGI at 18ºC)

Source	*df*	*SS*	*MS*	*F Ratio*	*Prob > F*
Model	2	46.36	23.18	91.17	<.0001
Error	59	15.00	0.25		
C. Total	61	61.35			

Effect test

Source	Nparm	*df*	*SS*	*F Ratio*	*Prob > F*
18 sex	1	1	0.20	0.81	0.37
18 time	1	1	46.35	182.32	<.0001

Analysis of CoVariance (Greenlip VGI at 12ºC)

Source	*df*	*SS*	*MS*	*F Ratio*	*Prob > F*
Model	2	6.18	3.09	25.29	<.0001
Error	47	5.74	0.12		
C. Total	49	11.92			

Effect test

Source	Nparm	*df*	*SS*	*F Ratio*	*Prob > F*
12 sex	1	1	0.02	0.18	0.68
12 time	1	1	6.18	50.58	<.0001

Analysis of CoVariance (Greenlip VGI at 14ºC)

Source	*df*	*SS*	*MS*	*F Ratio*	*Prob > F*
Model	2	3.81	1.90	22.48	<.0001
Error	38	3.22	0.08		
C. Total	40	7.02			

Effect test

Source	Nparm	*df*	*SS*	*F Ratio*	*Prob > F*
14 sex	1	1	0.04	0.51	0.48
14 time	1	1	3.77	44.53	<.0001

Analysis of CoVariance (Greenlip VGI at 16ºC)

Source	*df*	*SS*	*MS*	*F Ratio*	*Prob > F*
Model	2	52.49	26.25	166.30	<.0001
Error	87	13.73	0.16		
C. Total	89	66.22			

Effect test

Source	Nparm	*df*	*SS*	*F Ratio*	*Prob > F*
16 sex	1	1	0.27	1.68	0.20
16 time	1	1	52.47	332.48	<.0001

Analysis of CoVariance (Greenlip VGI at 18ºC)

Source	*df*	*SS*	*MS*	*F Ratio*	*Prob > F*
Model	2	20.01	10.01	49.71	<.0001
Error	59	11.88	0.20		
C. Total	61	31.89			

Effect test

Source	Nparm	*df*	*SS*	*F Ratio*	*Prob > F*
18 sex	1	1	0.19	0.97	0.33
18 time	1	1	18.29	90.87	<.0001

Appendix 2.

Analysis of CoVariance (Blacklip MGBI at 12ºC)

Source	*df*	*SS*	*MS*	*F Ratio*	*Prob > F*
Model	2	736.83	368.41	63.86	<.0001
Error	26	150.00	5.77		
C. Total	28	886.83			

Effect test

Source	Nparm	*df*	*SS*	*F Ratio*	*Prob > F*
12 sex	1	1	6.84	1.19	0.2861
12 time	1	1	715.14	123.95	<.0001

Analysis of CoVariance (Blacklip MGBI at 14ºC)

Source	*df*	*SS*	*MS*	*F Ratio*	*Prob > F*
Model	2	885.74	442.87	64.07	<.0001
Error	42	290.31	6.91		
C. Total	44	1176.05			

Effect test

Source	Nparm	*df*	*SS*	*F Ratio*	*Prob > F*
14 sex	1	1	21.85	3.16	0.0826
14 time	1	1	885.09	128.05	<.0001

Analysis of CoVariance (Blacklip MGBI at 16ºC)

Source	*df*	*SS*	*MS*	*F Ratio*	*Prob > F*
Model	2	1789.25	894.625	169.353	<.0001
Error	57	301.107	5.283		
C. Total	59	2090.35			

Effect test

Source	Nparm	*df*	*SS*	*F Ratio*	*Prob > F*
16 sex	1	1	11.2784	2.135	0.1495
16 time	1	1	1728.32	327.172	<.0001

Analysis of CoVariance (Blacklip MGBI at 18ºC)

Source	*df*	*SS*	*MS*	*F Ratio*	*Prob > F*
Model	2	2472.88	1236.44	86.81	<.0001
Error	57	811.85	14.24		
C. Total	59	3284.73			

Effect test

Source	Nparm	*df*	*SS*	*F Ratio*	*Prob > F*
18 sex	1	1.00	37.24	2.61	0.1114
18 time	1	1.00	2472.85	173.62	<.0001

Analysis of CoVariance (Greenlip MGBI at 12ºC)

Source	*df*	*SS*	*MS*	*F Ratio*	*Prob > F*
Model	2	665.38	332.69	86.32	<.0001
Error	39	150.31	3.85		
C. Total	41	815.68			

Effect test

Source	Nparm	*df*	*SS*	*F Ratio*	*Prob > F*
12 sex	1	1	10.45	2.71	0.1076
12 time	1	1	643.55	166.98	<.0001

Analysis of CoVariance (Greenlip MGBI at 14ºC)

Source	*df*	*SS*	*MS*	*F Ratio*	*Prob > F*
Model	2	1009.38	504.69	278.42	<.0001
Error	34	61.63	1.81		
C. Total	36	1071.01			

Effect test

Source	Nparm	*df*	*SS*	*F Ratio*	*Prob > F*
14 sex	1	1	0.28	0.15	0.6983
14 time	1	1	1008.37	556.28	<.0001

Analysis of CoVariance (Greenlip MGBI at 16ºC)

Source	*df*	*SS*	*MS*	*F Ratio*	*Prob > F*
Model	2	2550.45	1275.22	313.55	<.0001
Error	55	223.68	4.07		
C. Total	57	2774.13			

Effect test

Source	Nparm	*df*	*SS*	*F Ratio*	*Prob > F*
16 sex	1	1	64.6044	15.8851	0.0002
16 time	1	1	2440.304	600.0261	<.0001

Analysis of CoVariance (Greenlip MGBI at 18ºC)

Source	*df*	*SS*	*MS*	*F Ratio*	*Prob > F*
Model	2	1465.25	732.63	141.55	<.0001
Error	47	243.26	5.18		
C. Total	49	1708.52			

Effect test

Source	Nparm	*df*	*SS*	*F Ratio*	*Prob > F*
18 sex	1	1	19.59	3.79	0.04
18 time	1	1	1464.34	282.92	<.0001