FRDC FINAL REPORT

ABALONE AQUACULTURE SUBPROGRAM: THE COMMERCIAL CONTROL OF SPAWNING IN TEMPERATE ABALONE

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2000/204 Abalone Aquaculture Subprogram: The commercial control of spawning in temperate abalone

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OBJECTIVES:

1. To determine the biological zero point (BZP - the critical temperature below which there is no gonad development) for blacklip and greenlip abalone, and the relationship between temperature and gonad development.
2. To identify the temperature required to condition abalone over a set period of time.
3. To develop protocols for the commercial control of spawning in abalone by temperature manipulation.

OUTCOMES ACHIEVED

1. Hatchery managers have utilised the information from this study to predictably condition and spawn wild-caught blacklip and greenlip abalone broodstock.
2. The industry adoption of these procedures provides a reliable and continuous supply of high quality larvae vital for the consistent production of seedstock for abalone farming in southern Australia.

OUTPUTS

1. The BZP (a parameter used to determine the duration of gonad development) was estimated in wild-caught blacklip and greenlip abalone broodstock.
2. The optimum temperature for conditioning of both species was determined.
3. Broodstock conditioning and induction protocols were developed for the predictable spawning during and outside the normal breeding season. These protocols will be of benefit to the abalone farming industry in southern Australia.

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 on 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. Broodstock must also be fed a high quality diet in amounts slightly in excess of their needs.

Temperature is the main factor influencing rate of gonad development in most species. Its effect is cumulative above the BZP, which varies between species. We held animals from spent condition at 12°C, 14°C, 16°C or 18°C and routinely examined gonad development. The Visual Gonad Index (VGI) and Modified Gonad Bulk Index (MGBI) described gross

gonad structure while ovarian microstructure was described by oocyte volume, and rates of increase in these descriptors were used to estimate BZP. The BZP estimates based on VGI were 7.8°C for blacklips and 6.9°C for greenlips, and identical within species for males and females. BZP estimates for oocyte volume were similar (7.6°C and 6.8°C) but were up to 1.8°C lower (6.0°C for blacklips and 5.7°C for greenlips) and more inaccurate for MGBI. The BZP (from VGI) was used to calculate the Effective Accumulative Temperature (in degree days; EATºC-days), the difference between holding temperature and BZP, for each species at 16ºC or 18ºC for intervals ranging from 114-235 days before spawning induction with ultraviolet irradiation of the water followed by a second conditioning interval of the same length.

For blacklips, mean spawning rate of both sexes was higher at 18ºC than 16ºC, as was repeat spawning rate, whereas these parameters were higher at 16ºC for greenlips. However, for both species, egg and sperm production were higher for males and females at 16ºC than 18ºC. The recommended duration of conditioning blacklips at 16ºC is ≥188 days (≥1540 EATºC-days) for males and ≥165 days (≥1350 EATºC-days) for females. Corresponding figures for greenlips are ≥188 days (≥1700 EATºC-days) for males and ≥212 days (≥1930 EATºC-days) for females. However, there seems to be considerable flexibility in the time that blacklips and greenlips may be conditioned on formulated feeds for acceptable spawning performance for hatchery production. Males and females of both species mostly produced large numbers of gametes when induced to spawn following both the first and second conditioning intervals.

This study demonstrates that year-round hatchery production of seedstock of both species is possible providing broodstock are held under favourable environmental conditions, preferably 16ºC, and fed a formulated feed. Entraining animals of either species at 16ºC to a spawning cycle based on the recommended conditioning intervals would allow hatcheries to consistently produce large numbers of eggs and sperm for use in seed production. 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. 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.

It is likely that, with genetic improvement of hatchery-reared animals, the reliance on wild-caught animals will diminish. In the meantime, improving the production of gametes from captured animals is essential. The present study showed that adult blacklips and greenlips caught from the wild could be conditioned in the hatchery to produce reliable quantities of high-quality eggs and sperm. Further, conditioning can be conducted repeatedly with a high proportion of animals spawning and large numbers of gametes produced at each induction. Animals may now be better managed to spawn as required to meet the demand for juveniles (spat) for farming as well as to take advantage of unseasonal demands for earlier-than-normal production e.g. to allow a longer growing period over early summer which would increase survival and increase animal weights before entering the following winter. The selection of individuals in genetic improvement programmes, hybridisation between species and triploidy induction requires synchronisation of spawning which can now be better coordinated with the control of reproduction in conditioned animals.

KEYWORDS: *Haliotis*, blacklip, greenlip, abalone, gonad, reproduction, conditioning, temperature, biological zero point.

3 ACKNOWLEDGEMENTS

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4 BACKGROUND

The wild abalone fishery has historically supplied product to meet the international demand but in recent years aquaculture has helped to supply the product shortfall. Worldwide aquaculture production in 2002 was over 8,300 t, equal to 75% of the wild-caught production of 10,385 t, with the highest farmed production from China (4,500 t) and Taiwan (3,000 t) (Fleming, 2003). Farmed abalone production in Australia is growing rapidly, from 66 t in 2002, worth about $3 million, to an anticipated output of 550 t worth $24.5 million by 2006/7. The capacity of Australia's abalone aquaculture industry to capture a greater share of the expanding international market is dependent on a high degree of production efficiency and maximising product quality.

There are established farms for two temperate species, the blacklip abalone (*Haliotis rubra*) and greenlip abalone (*H. laevigata*) in Tasmania, South Australia, Victoria and Western Australia. These species and others (e.g. tropical abalone, *H. asinina*) are also under investigation for aquaculture in other States. Many farms are now expanding growout facilities after undertaking extensive research into the management of the production cycle, system design and nutrition. Research on growout systems and diets have achieved considerable increases in productivity which have been adopted by industry. However, it is recognised that unreliable spawning of broodstock is a bottleneck in production at the hatchery level and needs to be solved to facilitate expansion of the industry.

At present, hatchery production of abalone larvae generally relies on the capture and induced spawning of wild broodstock but the importance of cultured broodstock will increase in future. Even though techniques for the hatchery rearing of abalone are well developed in Australia, broodstock conditioning (stimulation of sexual maturation) of wild-caught abalone is poorly understood and inconsistent. Even for animals at the seemingly right stage of maturity, there are difficulties in inducing spawning at the desired time. These problems have hindered the production from hatcheries and restricted the expansion of the industry.

Inconsistent gamete and larval production has led to considerable fluctuations in the quantity of seedstock available for growout. Ensuring that individual broodstock spawn predictably would increase hatchery efficiency and allow for out-of-season production. This, in turn, allows hatcheries to take advantage of seasonality in peak growing conditions. The ability to spawn selected individuals will also aid genetic improvement programmes aimed at increasing farm productivity.

It is advantageous to optimise the growth and survival of young abalone by transferring them to nursery areas in early spring. This improves survival and growth in the first winter. However, it is difficult to find sexually mature wild broodstock at this time and consequently insufficient larvae are produced. Producing larvae from broodstock that are matured out-of-season will take advantage of the longer growing season. There is also considerable commercial interest in culturing the hybrid between blacklips and greenlips as it has a number of production advantages over pure species lines. However, the difficulty of synchronising the spawning between species frustrates the development of this sector of abalone farming. Control of reproduction will allow synchronous spawning between the two species.

Temperature is considered the prime trigger for gonadal development for most species of abalone (although it is the response is modulated to a large degree by nutrition i.e. feed composition, feed ingestion rate). However, full quantification of the requirements for gonad

conditioning remains unknown for most species. Some abalone seem to have a simple response to temperature that is cumulatively additive in terms of reproductive conditioning (e.g. *H. discus hannai, H. australis*) while other species may be more complex (e.g. *H. gigantea*). Although it is likely that both blacklips and greenlips have a simple response to temperature, this has not yet been determined and is the subject of this study.

The Abalone Aquaculture Subprogram identified the issue of broodstock conditioning as requiring immediate resolution to satisfy problems in larval production, but in the longer term to allow genetic improvement to be undertaken. This project investigated the influence of water temperature on the control of spawning in abalone (blacklip, greenlip) for the reliable and efficient hatchery production of larvae at commercially optimum times. It is anticipated that better control will improve the predictability of spawning, the synchrony of spawning between sexes, species and selected individuals, and the rates of fecundity, while maintaining gamete quality.

The project examined methodologies for the control of spawning that will be adopted by industry. The temperature manipulations, animal husbandry and protocols were commercially oriented, both practically and economically. The project also examined some of the fundamental aspects of the biology of abalone reproduction to provide the physiological understanding for the control of spawning.

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Fleming, A.E., 2003. Final report of FRDC project no. 2000/200: Abalone Aquaculture Subprogam: Facilitation, administration and promotion. In: Fleming, A.E. (Ed.), Proceedings of the 10th Annual Abalone Aquaculture Workshop. Port Lincoln, South Australia. 39 pp. Fisheries Research and Development Corporation, Abalone Aquaculture Subprogram. Canberra, Australia.

5 NEED

Protocols for the commercial control of spawning have been identified by industry as essential for consistent larval production and for genetic improvement. Typical problems in controlling the spawning of abalone broodstock include:
- wild broodstock in an advanced stage of gonad development spawn unreliably or do not spawn at all when induced.
- inability to access gravid broodstock outside the natural spawning season.
- variability in gamete quality and quantity of young cultured broodstock.

Unexpected shortages in the supply of juveniles for ongrowing lead to reduced efficiency for the entire production phase. Attempting to spawn non-responsive wild-caught adults in apparently advanced gonad condition may frustrate the hatchery phase and the entire production season. It is therefore vital that wild-caught animals be conditioned to meet the hatchery requirements for gametes.

Early spawning or out-of-season spawning allows farmers to take advantage of a longer growing period for young juveniles. The relatively larger juveniles are then more resilient to cold water and so survive and grow better during their first winter. This necessitates that broodstock be ready to spawn as spring water temperatures begin to increase.

6 OBJECTIVES

1. To determine the biological zero point (BZP) and the relationship between temperature and gonad development.
2. To identify the temperature required to condition abalone over a set period of time.
3. To develop protocols for the commercial control of spawning in abalone by temperature manipulation.

7 TEMPERATURE EFFECTS ON THE DYNAMICS OF GONAD AND OOCYTE DEVELOPMENT IN CAPTIVE WILD-CAUGHT BLACKLIP (*HALIOTIS RUBRA*) AND GREENLIP (*H. LAEVIGATA*) ABALONE

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7.1 Abstract

Wild-caught blacklip (*Haliotis rubra*) and greenlip (*H. laevigata*) abalone were held from spent condition at 12°C, 14°C, 16°C or 18°C 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 gonad development occurs. BZP estimates derived from the daily increase in VGI and oocyte volume were similar (7.8°C and 7.6°C for blacklips; 6.9°C and 6.8°C for greenlips, respectively), but those based on the increase in MGBI were up to 1.8°C lower (6.0°C and 5.7°C, for blacklips and greenlips, 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 spawning of these species. Knowledge of the EAT facilitates predictive and deductive estimates of spawning events (when water temperature is known) and comparison of spawning patterns both within and between species.

Keywords: Biological zero point; *Haliotis laevigata*; *Haliotis rubra*; Modified gonad bulk index; Oocyte morphometrics; Temperature; Visual gonad index

7.2 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* at different water temperatures. The VGI is a non-invasive, semi-quantitative means of assessing gonad bulk in abalone and usually 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, there were positive linear relationships between the VGI (when the mean VGI $\leq$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 spawning. At present, there is only one account of the EAT for spawning of southern hemisphere abalone species (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 Clark (1934). However, oocyte size frequency polygons are often difficult to interpret and cannot be used for 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 firstly to 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 secondly 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 effective accumulative temperature for spawning of these species (when the daily temperature and the interval between successive spawnings is known). This allows comparisons of spawning patterns 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.

7.3 Methods

7.3.1 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 zero. Animals with VGI values of 2 or 3 were induced to spawn using UV-irradiated seawater (Grubert and Ritar, 2002). Only animals that spawned or had zero 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 the spionid polychaete mudworm *Boccardia knoxi*.

7.3.2 Experimental design

Abalone of each sex and species were held in 150 L fibreglass tanks. Within each sex and species group, 36 animals were randomly assigned to each of four experimental temperatures (12, 14, 16, 18°C) with two replicate tanks per temperature treatment (2 species x 2 sexes x 4 treatments x 2 replicates = 32 tanks total). The time interval for sampling was based on estimated BZP figures of 7.0°C for blacklip abalone and 8.0°C for greenlip abalone and an EAT interval of 400°C-days for both species. For blacklip abalone, the number of days between each sampling (n = the number of samplings at each temperature) were 80 days at 12°C (n = 3), 57 days at 14°C (n = 4), 44 days at 16°C (n = 6) and 36 days at 18°C (n = 6). Corresponding values

for greenlip abalone were 100 days at 12°C (n = 3), 67 days at 14°C (n = 4), 50 days at 16°C (n = 6) and 40 days at 18°C (n = 60). At these times, VGI was determined in 4-6 animals in each treatment group (selected at random from the duplicate 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).

7.3.3 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.

7.3.4 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. 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.

7.3.5 Calculation of the Modified Gonad Bulk Index (MGBI) 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. 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 was 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 ($max_r = min_{Ø}/2$) 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 $min_{Ø}$.

7.3.6 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 identical conditions, it was assumed that they were at the same stage of development when sampled. 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.

7.4 Results

7.4.1 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, 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 7.1a and 7.1b).

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, but the data were pooled for this analysis. The mean MGBI increased linearly during conditioning in both species (Fig. 7.2) with values ranging from 5–68 mm^3g^{-1} and 5–58 mm^3g^{-1} for blacklips and greenlips, respectively. The low r^2 values for the regressions between MGBI and shucked weight for blacklip (0.02) and greenlip abalone (0.01) confirm that MGBI is a size independent measure of gonad bulk in these species.

7.4.2 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 related to 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 7.3a-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. 7.3c, it can be seen that 100% vitellogenesis occurs in blacklip oocytes >90 µm (L. Gurney, unpublished data), while Fig. 3d shows that the size frequency histogram for greenlip oocytes (this study) is similar in form.

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 7.1) is further evidence that EV provides a better estimate of oocyte volume than SV.

Oocyte volume increased as temperature and conditioning time increased (Figs 7.4a and 7.4b) 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.

7.4.3 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. 7.5a-c) and 0.99, 0.99 and 0.99 for greenlips (GL, Figs. 7.5d-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 7.2).

7.4.4 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 7.3 and 7.4 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.

7.5 Discussion

7.5.1 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. In the case of *H. rubra* this figure was 7.8ºC, which is similar to that reported for *H. discus hannai* (7.6ºC; Kikuchi and Uki, 1974a). The BZP estimate for *H. laevigata* (6.9ºC) is comparable to that of *H. 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* (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 would stress some (or all) individuals, which may result in gonad development being "deferred" until water temperature falls back 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 gonad. 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, 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 abalone, 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, be it at one time or over the course of a year. 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).

7.5.2 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 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 (1994) work based on 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.

7.6 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 temperature below which no gonadal development occurs. The most reliable estimates of BZP, based on VGI, were 7.8ºC for blacklips and 6.9ºC for greenlips. We recommend that conditioning time should be based on the BZP and expressed as the EAT in degree days to better predict the duration of gonad conditioning. 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, in future, contingency table analysis of oocyte size frequency in abalone should use ellipsoid volume paying particular attention to sampling frequency and categorization of oocyte size classes.

7.7 Acknowledgements

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Table 7.1. 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

Table 7.2. 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

Table 7.3. 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 ($\mu m^3 \times 10^5$)									
		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 7.4. 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 ($\mu m^3 \times 10^5$)									
		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**

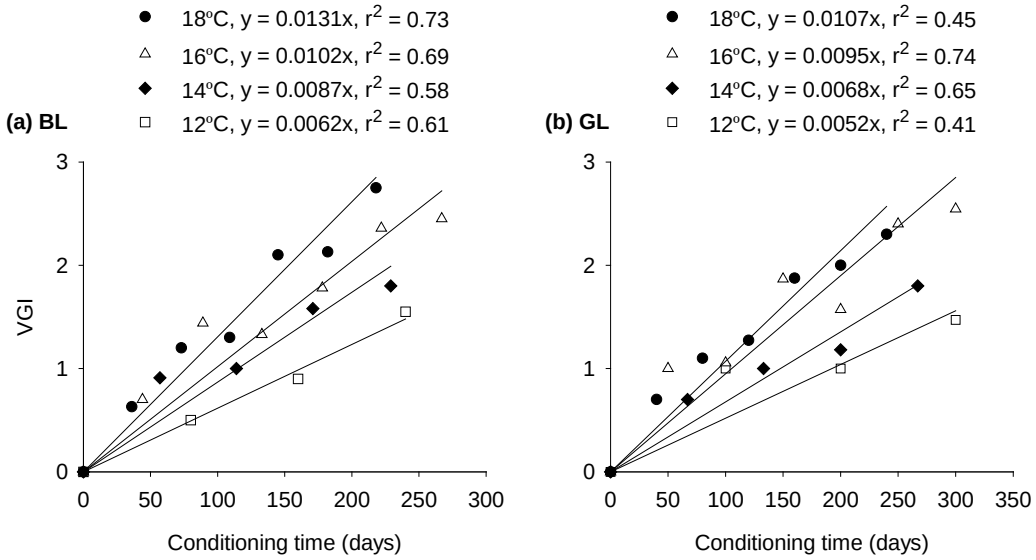

Fig. 7.1. Increase in mean Visual Gonad Index (VGI) score relative to conditioning time and culture temperature in blacklip (BL, a) and greenlip (GL, b) abalone. Data for males and females within species were pooled.

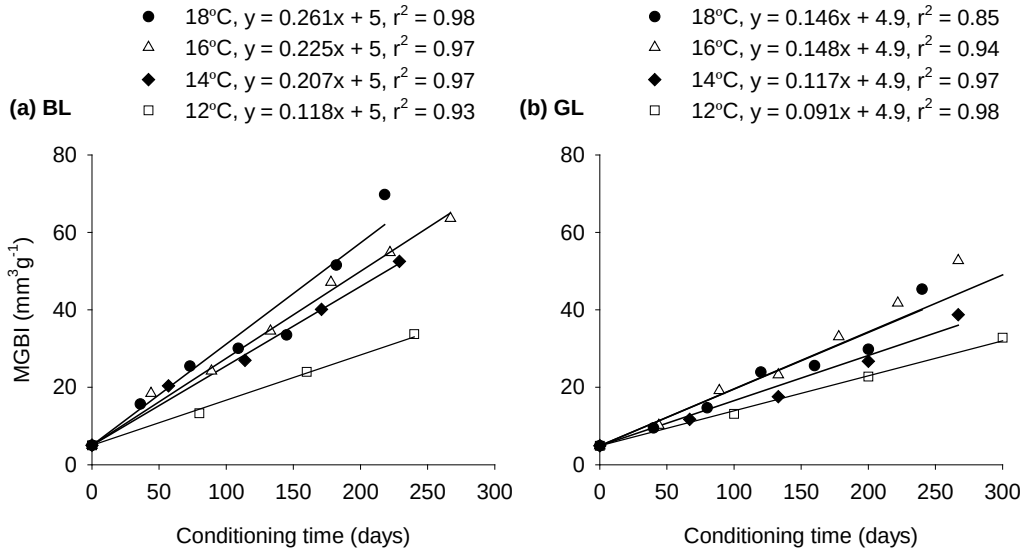

Fig. 7.2. Increase in Modified Gonad Bulk Index (MGBI) relative to conditioning time and culture temperature in blacklip (BL, a) and greenlip (GL, b) abalone. Lines for the greenlip 16ºC and 18ºC treatments overlap. Data for males and females within species were pooled.

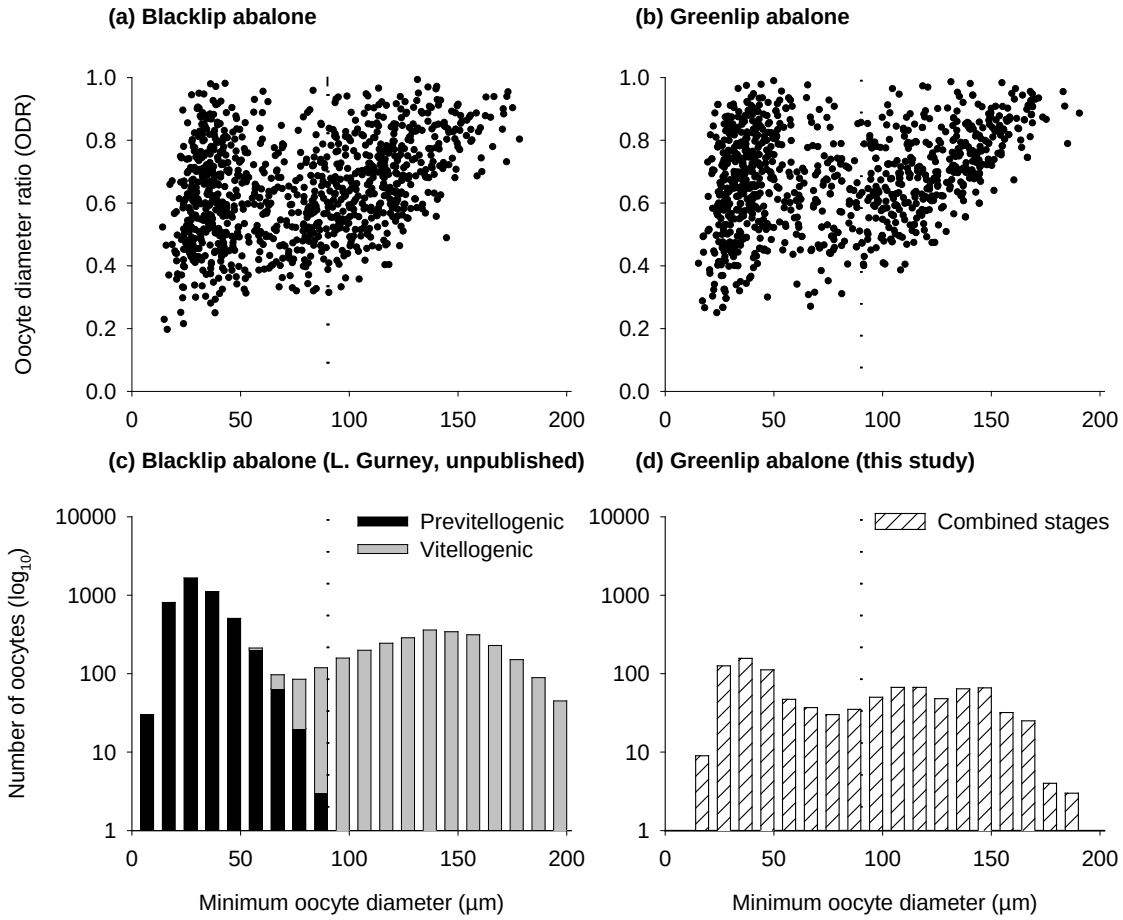

Fig. 7.3. The relationship between minimum oocyte diameter and Oocyte Diameter Ratio (ODR; minimum diameter / maximum diameter) in (a) blacklip and (b) greenlip abalone as well as stage and size frequency of oocytes in (c) blacklip (from L. Gurney, unpublished) and (d) greenlip abalone (this study). Dashed lines indicates minimum oocyte diameter of 90 µm.

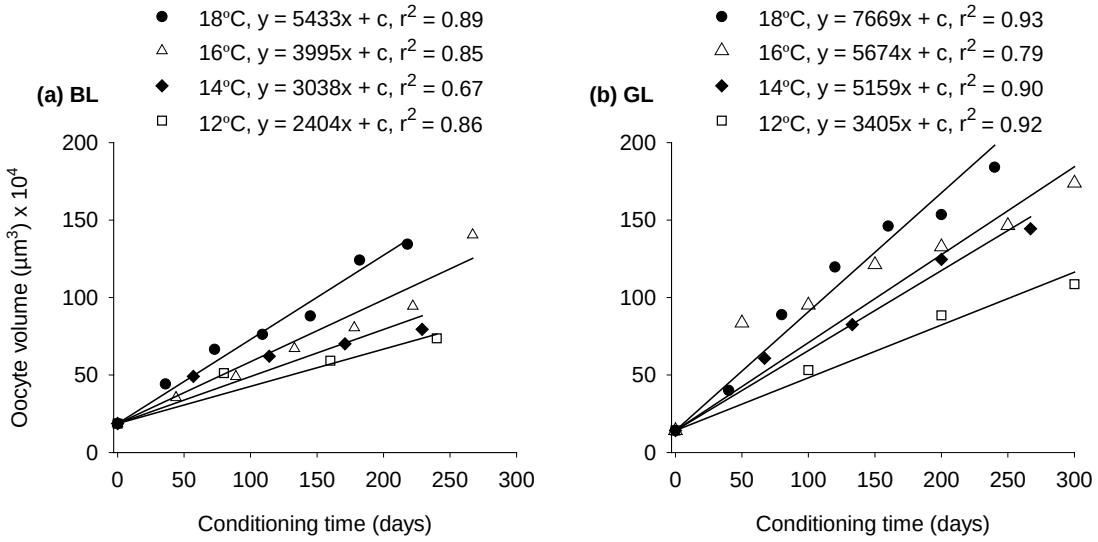

Fig. 7.4. The relationship between conditioning time (x), culture temperature and oocyte volume (y) in (a) blacklip and (b) greenlip abalone. Values of constant c were 1.86×10^4 and 1.42×10^4 for blacklip (BL) and greenlip (GL) abalone, respectively.

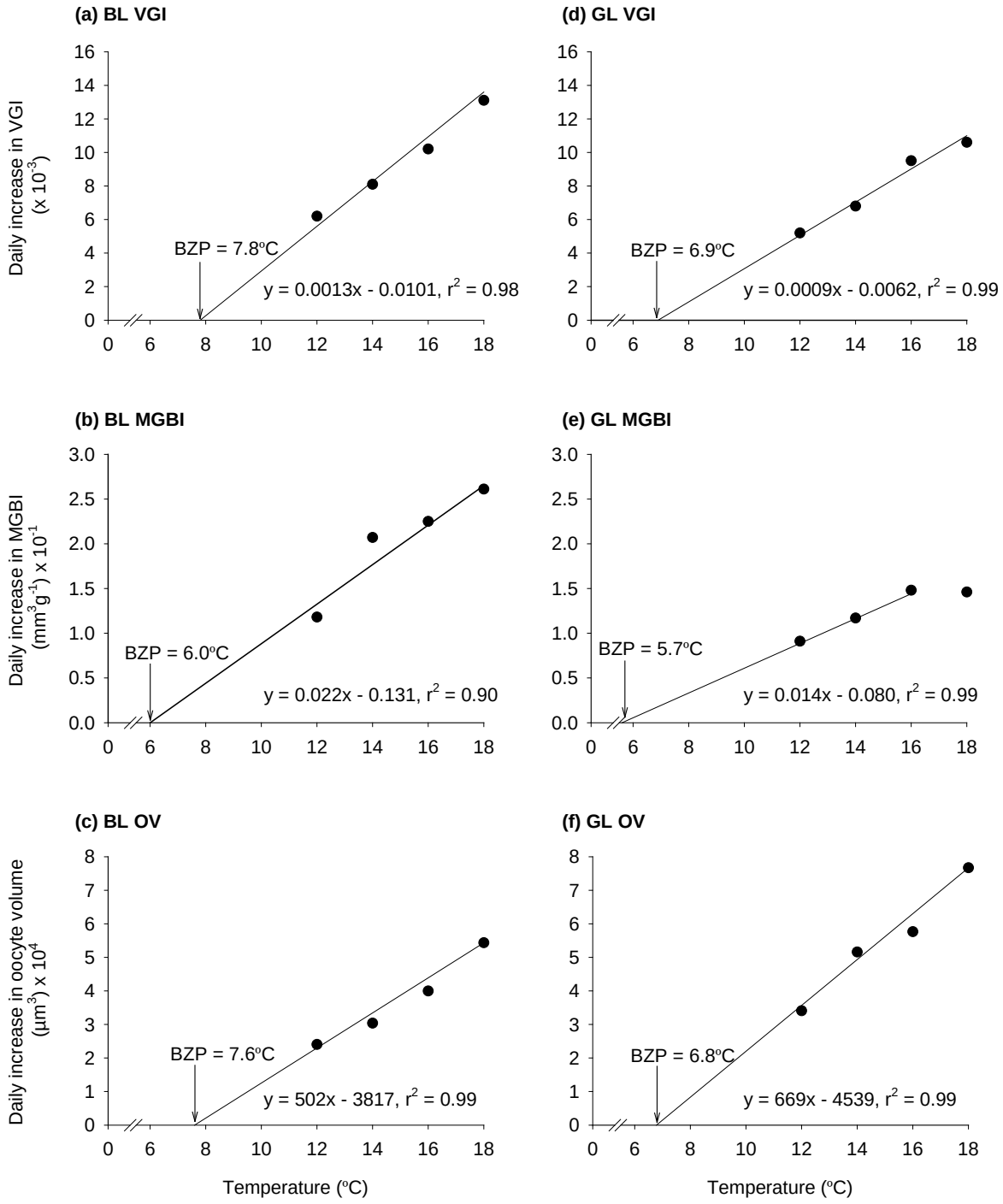

Fig. 7.5. The relationship between Visual Gonad Index (VGI), Modified Gonad Bulk Index (MGBI), oocyte volume and culture temperature in blacklip (BL, a–c) and greenlip (GL, d–f) abalone. Linear relationship in 5e did not include the outlier value at 18ºC.

8 THE EFFECT OF TEMPERATURE AND CONDITIONING INTERVAL ON THE SPAWNING SUCCESS OF WILD-CAUGHT BLACKLIP (*HALIOTIS RUBRA*) AND GREENLIP (*H. LAEVIGATA*) ABALONE FED AN ARTIFICIAL DIET

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8.1 Abstract

Wild-caught blacklip (*Haliotis rubra*) and greenlip (*H. laevigata*) abalone fed an artificial diet were held at 16ºC or 18ºC for different conditioning intervals (calculated as Effective Accumulative Temperature, EAT, a function of the number of days that animals were held at each temperature) ranging from 1160–2140 EATºC-days. Ten to fifteen animals of each sex and species were individually placed in separate trays and induced to spawn using ultraviolet-irradiated seawater. They were conditioned again for a second identical period before another induction. Spawning rate, repeat spawning rate (i.e. animals spawning at both inductions), mean and total gamete production were used as indicators of spawning performance. 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. Mean egg production by *H. rubra* was 0.3–2.1 x 10^6 female^{-1}, and total production 1.0–11.1 x 10^6 group^{-1}. Egg production peaked in groups held at 16ºC for ≥1350 EATºC-days. While both mean and total sperm production of *H. rubra* varied significantly, both figures were always high (1.1–6.0 x 10^{11} male^{-1} and 10.9–71.5 x 10^{11} group^{-1}, 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. Mean egg production was 0.1–1.9 x 10^6 female^{-1}, and total egg production 0.3–15.5 x 10^6 group^{-1}. Egg production peaked in groups conditioned at 16ºC for ≥1930 EATºC-days due to the high proportion (>60%) of animals spawning at these inductions. Both mean and total sperm production by *H. laevigata* was much lower than for *H. rubra* and varied considerably (0.006–2.7 x 10^{11} male^{-1} and 0.03–28.5 x 10^{11} group^{-1}, respectively), as did the spawning rate (25–100%). By contrast to all other groups, mean gamete production by male *H. laevigata* improved at the second induction, in some cases increasing by two orders of magnitude. Neither culture temperature nor conditioning interval had a significant effect on egg fertilization or larval hatchout rates in either species. This study demonstrates that year-round hatchery production of seedstock of both species is possible providing broodstock are held under favourable environmental conditions, preferably at 16ºC.

Keywords: *Haliotis rubra*; *Haliotis laevigata*; Temperature; Broodstock conditioning; Induced spawning

8.2 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 on 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. Broodstock must also be fed a high quality diet in amounts slightly in excess of their needs (Uki and Kikuchi, 1982a).

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 (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 abalone, that of Kabir (2001) for *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 16°C but did not examine spawning success. Plant (2002) conditioned *H. laevigata* at 17°C 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 5°C over this interval showed the best results. Savva et al. (2000) examined the effect of broodstock diet on fecundity and egg quality in *H. rubra* conditioned at 16°C. Broodstock fed an artificial diet 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 et al. (2002) documented the spawning success of groups of blacklip abalone held at 18°C 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.

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 an artificial diet. This was achieved by holding both sexes of each species at temperatures of either 16°C or 18°C for one of five different conditioning intervals (calculated as EAT°C-days) over two conditioning cycles. The optimal conditioning 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 quality and quantity.

8.3 Materials and methods

8.3.1 Broodstock collection

Greenlip abalone broodstock (100–120 mm shell length - SL) 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 SL) 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 et al., in press; 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, 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. Most appeared healthy although some greenlips had small numbers (< 20) of the shell parasite, *Boccardia knoxi*, also known as mudworm. While the infestation level of each abalone was not monitored, the abundance of mudworm did not appear to change during the course of the experiment.

8.3.2 Experimental design

At the start of the experiment, 10 to 15 abalone of each sex and species were randomly assigned to one of two temperatures (16°C or 18°C) and five conditioning intervals (1200, 1400, 1600, 1800 or 2000 EATºC-days, referred to in the text as 1200 group, 1400 group etc.). The EAT was calculated on the basis of the estimated biological zero point (BZP) of 7.5°C for both species. The BZP was subtracted from the actual holding temperature (e.g. 16-7.5 = 8.5°C or 18-7.5 = 10.5°C) 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 and Ritar, in prep) (see Chapter 7).

For each sex, species and temperature treatment there were three tanks (2 x 2 x 2 x 3 = 24 tanks in total). One contained the 1200 and 1400 groups, another the 1600 and 1800 groups and a third, the 2000 group and 12 other animals to maintain equal density. When the conditioning interval for the group elapsed, they were induced to spawn, reconditioned for the same interval and induced again.

8.3.3 Husbandry and monitoring

Broodstock were conditioned in 150 L round fibreglass tanks (approximately 25 animals $tank^{-1}$) receiving flow-through seawater at a rate of 1.5 L min^{-1} $tank^{-1}$. Animals were fed daily to satiation on a broodstock conditioning diet (Adam and Amos Abalone Foods Pty Ltd, South 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, Massachusetts).

8.3.4 Induction of spawning

On each induction day, abalone were removed from their holding tanks at 09:00h. The VGI of each animal was recorded prior to being placed into one of twenty 14 L polyethylene spawning trays containing 10 L seawater. In some cases these trays were partitioned with plastic panels so as to hold two animals separately. Water temperature in the trays was the same as the conditioning temperature (i.e. 16°C or 18°C). Female abalone were placed in the top row of 10 trays and males in the bottom row. 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, New South Wales), the quartz sleeves of which were cleaned after every 50 hours of use. Water temperature was raised by 1°C h^{-1} for 4 h then allowed to return to the original temperature at the same rate (i.e. 16°C→20°C→16°C or 18°C→22°C→18°C). Flow rate to each upper tray was 300 ml per minute. The water then cascaded to the tray below. Animals were checked every 15 min from 14:00–22:00h and the time that they began spawning recorded. Initiation of spawning was not recorded after 22:00h.

When males spawned prior to 22:00h, sperm water was poured off, made up to a known volume, mixed and samples taken for motility and density (using absorbance at 340 nm, Ritar and Grubert, 2002) estimates and fertilization trials. This was done within 1 h of initiation of spawning. 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 in triplicate to estimate fecundity. When sperm was collected from the same male on two occasions (at night and the following morning), sperm production estimates were summed.

8.3.5 Gamete quality

When each group was induced the second time, gametes from a random selection of males and females that spawned prior to 20:00h were collected to assess sperm motility, egg fertilization and larval hatchout rates (it was not logistically possible to run further trials past 20:00h). The percentage progressively motile sperm was assessed under a compound microscope to the nearest 5%. To assess fertilization success, approximately 1000 eggs from each female was inseminated using highly motile sperm (< l h old, from a single male) at a concentration of 1.0 x 10^6 ml^{-1} and cultured in 70 ml polyethylene jars containing 50 ml of 1µm-filtered seawater and 25 ppm oxytetracyline (OTC). OTC was used to avoid bacterial contamination. Fifteen minutes post insemination (PI), the sperm were rinsed off and a new solution of seawater and OTC added. The jars were then placed in a water bath at 18ºC. Fertilization rate was recorded at 2 h PI and hatchout rate at 20 h PI.

8.3.6 Statistics

For both sexes of each species, analyses of gamete production and spawning response time data were conducted using one-way (with unequal variance) 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. Probabilities of <0.05 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 (i1) then at the second induction (i2), using data from only those animals that had spawned. A second analysis examined how total gamete production at both inductions (i.e. x_i = i1 + i2, 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. Data used in both these tests were √transformed prior to analysis (Sokal and Rohlf, 1995). Due to lack of independence in data from successive inductions, no direct comparisons of the first and second inductions were made. However, the difference in gamete production between the first and second inductions was calculated for each individual that spawned at least once (x_i = i2 - i1) and standardized by adding the absolute value of the largest negative value (x_{min}) plus one (ie. $x_i = x_i + |x_{min}| + 1$). These data were analyzed in the same manner as above but not √transformed and are referred to as IndDiff in the text. One-way ANOVA was employed to test for differences in mean spawning rates and mortality rates between each temperature treatment (% 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 SL and gamete production. Statistics were executed using Excel 2000 (Microsoft) and JMP version 5.0 (SAS Institute Inc.).

8.4 Results

8.4.1 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 were 49 ± 4% and 63 ± 3%, respectively (n.s., Table 8.1). Mean spawning response times were 6h 32min at 16ºC and 6h 05min at 18ºC (n.s., Table 8.2). Maximum individual egg production at 16ºC and 18ºC was similar (3.8×10^6 and 3.9×10^6 eggs, respectively), as was maximum group production (10.6×10^6 and 8.9×10^6 eggs, respectively). Minimum and maximum values for group mean egg production tended to be higher at 16ºC ($0.9–2.0 \times 10^6$ eggs group^{-1}) than 18ºC ($0.5–1.5 \times 10^6$ eggs group^{-1}), but the difference was not significant. Likewise, there was no EAT effect on this factor (Table 8.2).

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 (Table 8.1). Mean response times were similar; 7h 02min at 16ºC and 6h 27min at 18ºC. Mortalities were 5% at 16ºC and 16% at 18ºC (n.s., Table 8.2). Maximum individual egg production was similar at 16ºC and 18ºC (4.8×10^6 and 4.4×10^6 eggs, respectively), as were group totals (10.6×10^6 and 11.1×10^6, respectively). There was no significant relationship between SL and egg production ($r^2 = 0.001$).

There was a significant interaction between temperature and EAT conditioning interval 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 than the other groups. In the case of the animals held at 18ºC, the 1200 group produced significantly more eggs than all other groups. There were no significant temperature or EAT 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 (Table 8.2).

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

At the first induction, spawning rate of male blacklip groups in both temperature treatments was high (93 ± 5% and 97 ± 3% at 16ºC and 18ºC, respectively; n.s., Table 8.1). The mean spawning response times for each temperature group was similar; 6h 15min at 16ºC and 5h 45 min at 18ºC (Table 8.2). While this difference was not significant, there was a T*EAT interaction in spawning response times, but there was no obvious trend in the data. Maximum individual sperm production was similar at both temperatures (9.9 and 11.1×10^{11} sperm at 16ºC and 18ºC, respectively). The highest group production total for the 16ºC treatment was 71.5×10^{11} sperm (1800 group) and for the 18ºC treatment 52.5×10^{11} (1800 group). A significant temperature and EAT conditioning interval effect (at 16ºC) on group mean sperm production was detected (Table 8.2). Mean sperm production of EAT groups held at 16ºC ranged from 1.1–6.0 x 10^{11}, and that of the 18ºC groups from 1.2–4.8 x 10^{11}.

Spawning rate in both temperature treatments at the second induction was also high (83 ± 5% and 94 ± 4% at 16ºC and 18ºC, respectively; n.s., Table 8.1). The proportion of repeat spawners was 76 ± 9% at 16ºC and 93 ± 3% at 18ºC. 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 held at either temperature spawned twice. The proportion of repeat spawners was highest in the 18ºC group while the proportion of animals that spawned only once was highest in the 16ºC group. As for the first induction, there was a significant interaction between temperature and EAT conditioning interval on spawning response times when male blacklip abalone were induced a second time, but no obvious trend in the data was discernible (Table 8.2). Mortalities were low in both temperature groups (5% and 3% at 16ºC and 18ºC, respectively; n.s.).

Maximum individual sperm production was similar at 16ºC and 18ºC (9.8 and 10.5×10^{11} sperm, respectively) as were group production totals (66.0×10^{11} sperm and 65.2×10^{11}, respectively). There was a significant interaction between temperature and EAT conditioning interval on mean sperm production of animals at the second induction. Of the males held at 16ºC, only the 1200 and 1600 groups differed significantly (range of group means = 1.5–5.3 x

10^{11} sperm male^{-1}; Table 8.2) whereas at 18ºC, the 2000 group produced significantly more sperm than all other groups (range of group means = 1.2–5.9 x 10^{11} sperm male^{-1}). There was no significant relationship between SL and sperm production (r^2 = 0.001).

There was a significant T*EAT interaction on the means of the standardized difference in gamete production between the first and second inductions (IndDiff). However, the interaction was influenced by one group that showed a significantly greater increase in sperm production between inductions than all others, the 2000 group held at 18ºC (Table 8.2). An interaction between temperature and EAT conditioning interval on total gamete production over both inductions (IndTotal) was also detected. IndTotal increased proportionally with EAT, although this difference was largely driven by animals in the 16ºC groups. The 16ºC males produced more sperm than those in the 18ºC treatment, except for the longest and shortest conditioning intervals where the values were equal.

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

At the first induction, the mean spawning rate of female greenlip groups held at 16ºC was 56 ± 11% and at 18ºC was 37 ± 5% (n.s.; Table 8.3). Mean spawning response times were similar at both temperatures (8h 50min at 16ºC and 9h 14min at 18ºC) and there was no EAT effect on this variable (Table 8.4). At both temperatures, the 2000 group produced the highest egg total (15.1 x 10^6 and 2.6 x 10^6 eggs group^{-1} for the 16ºC and 18ºC treatments, respectively). The best individual spawner at 16ºC produced just over twice as many eggs as the best spawner at 18ºC (3.5 x 10^6 and 1.7 x 10^6 eggs, respectively). Significant temperature and EAT conditioning interval effects on mean egg production were detected. Females held at 16ºC produced more eggs than those held at 18ºC (Table 8.4). Mean egg production by groups held at 16ºC ranged from 0.4–1.7 x 10^6 eggs individual^{-1}, with the 1400 group producing significantly fewer eggs than other groups. In the 18ºC treatment, mean egg production ranged from 0.1–0.7 x 10^6 eggs individual^{-1} with no significant differences between groups.

When induced a second time, the mean spawning rate of groups at 16ºC was 68 ± 14% and at 18ºC was 64 ± 13% (n.s., Table 8.3). Mean repeat spawning rate was 47 ± 2% at 16ºC and 28 ± 9% at 18ºC. 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. As for the first induction, there was no temperature or EAT effect on mean spawning response times when the animals were induced a second time (Table 8.4). Mortality rates were 5% and 9% at 16ºC and 18ºC, respectively (n.s.). The maximum individual egg production at 16ºC (5.9 x 10^6 eggs) was more than double that at 18ºC (2.5 x 10^6 eggs). The highest group total at 16ºC was also 3 million greater than that at 18ºC (15.4 x 10^6 and 12.4 x 10^6, respectively). A significant temperature effect (but no EAT conditioning interval effect) on mean egg production was found at the second induction. Females held at 16ºC produced significantly more eggs than those held at 18ºC. Mean egg production by 16ºC groups varied from 1.3–1.9 x 10^6 eggs female^{-1} and in the 18ºC groups from 0.7–1.6 x 10^6 eggs female^{-1}. There was no significant relationship between SL and egg production (r^2 = 0.003).

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

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

At the first induction, mean spawning rate of male greenlip groups at 16ºC (77 ± 9%) was significantly higher than that of 18ºC groups (42 ± 10%; Table 8.3). There was no temperature or EAT effect on mean spawning response times. The best individual spawner at 16ºC produced 4.9 x 10^{11} sperm, twenty times more than the maximum individual value recorded at 18ºC (0.2 x 10^{11} sperm; Table 8.4). Comparisons of group totals showed a similar trend, with the maximum values for the 16ºC treatment (13.6 x 10^{11} sperm) almost 25 times greater than that for the 18ºC treatment (0.6 x 10^{11} sperm). A significant T*EAT interaction on the mean sperm production was detected. Sperm production was significantly higher for animals held at ≥1600 EATºC-days at 16ºC than remaining groups at 16ºC and all groups at 18ºC. Mean sperm production varied from 0.006–1.0 x 10^{11} sperm male^{-1} in groups at 16ºC and 0.008–0.08 x 10^{11} sperm male^{-1} in groups at 18ºC.

When induced a second time, the spawning rate of all male groups was high (84 ± 3% and 88 ± 5% for the 16ºC and 18ºC treatments, respectively; Table 8.3). The mean repeat spawning rate was 60 ± 7% for the 16ºC groups and 31 ± 12% for the 18ºC groups (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, there was a significant difference at 18ºC. At this temperature, 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 ranged from 6h 53min to 9h 24min in the 16ºC groups and 8h 19min to 9h 12min in the 18ºC groups (Table 8.4). There was a significant T*EAT interaction on spawning response times but no obvious trend in the data was discernible. Mortality rates were 16% and 13% at 16ºC and 18ºC, respectively (n.s.). Similar to the first induction, maximum individual and group production totals at 16ºC were several times greater than at 18ºC. The best individual spawner at 16ºC produced 7.0 x 10^{11} sperm, just over twice the corresponding value for the 18ºC treatment (3.2 x 10^{11} sperm). There was also a three-fold difference in maximum group production between the 16ºC and 18ºC treatments (28.5 x 10^{11} and 8.5 x 10^{11} sperm group^{-1}, respectively). A significant temperature effect (but no EAT conditioning interval effect) on mean sperm production was detected. Males held at 16ºC produced significantly more sperm than those held at 18ºC. Mean sperm production ranged from 0.6–2.7 x 10^{11} sperm male^{-1} at 16ºC and from 0.4–0.8 x 10^{11} sperm male^{-1} at 18ºC. There was no significant relationship between SL and sperm production (r^2 = 0.032).

A significant temperature effect (but no EAT conditioning interval effect) was detected in the mean values of the standardized difference in sperm production between the two inductions (IndDiff). Values of IndDiff for animals held at 16ºC were 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. There was no significant change in IndTotal across the range of conditioning intervals in 18ºC males.

8.4.5 Gamete quality

Sperm motility, egg fertilization and larval hatchout rates were only recorded during the second round of conditioning. Mean sperm motility of blacklip abalone ranged from 67–95% for animals held at 16ºC and 72–92% for those held at 18ºC (Table 8.5). Since sperm samples were taken at the same time during an induction, the sperm motility of males spawning earlier may have lowered the group mean as motility decreases over time. Nevertheless, the fertilization rate of blacklip abalone eggs was greater than 80% in all cases. Hatchout rates were generally within 5% of the fertilization rates.

Mean sperm motility of greenlip abalone ranged from 71–93% for animals held at 16ºC and 67–86% for those held at 18ºC. The fertilization rate of greenlip abalone eggs was always above 80%. Hatchout rates were generally within 5% of the fertilization rates, although in one case (the 1200 group at 18ºC) this difference was 18%.

8.5 Discussion

8.5.1 Spawning rate and gamete production relative to temperature and conditioning interval

Conditioning of blacklip and greenlip abalone on an artificial diet 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.

Although this study used an estimated BZP of 7.5ºC for both species, work conducted concurrently showed that the actual BZP for gonad development of these species was 7.8ºC and 6.9ºC for blacklips and greenlips, respectively (Grubert and Ritar, in prep). 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 8.6.

Both 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 ≥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) and ≥1930 EATºC-days (212 days) for males and females, respectively.

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 8.7). Plant et al. (2002) found that these parameters in female *H. rubra* peaked after 120 days conditioning at 18ºC (92% spawned; 2.0×10^6 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 (1.1×10^6 eggs female^{-1}). By contrast, no reduction in egg production by female *H. rubra* held for ≥1200 EATºC-days was observed during this study. There was no relationship between SL and egg (or sperm) production in *H. rubra*, most likely due to partial spawning and/or the narrow size range used.

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 8.7). Only 4% of females spawned more than once, suggesting that a conditioning cycle of 12 weeks (approximating 690 EAT ºC-days) was insufficient for oocyte development. 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 and Edwards, 1998), there may have been lasting adverse effects on broodstock spawning performance.

The maximum egg production estimate for *H. laevigata* reported here is greater than most estimates in previous works, with the exception of Babcock and Keesing (1999). These authors used larger animals (see Table 8.7) 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 SL 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 egg production rates of 0.1–1.0 x 10^6 eggs female^{-1}, while no sperm production was given. Interestingly, when this author induced small (60–80 mm SL), cultured *H. laevigata* to spawn, 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 8.7).

Plant (2002) studied the spawning performance of wild-caught *H. laevigata* that were held at 17ºC in a commercial hatchery. These animals were subjected to previous conditioning experiments and so were at different stages of maturity when induced. Consequently, spawning rate at the initial induction 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 recorded 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 (0.01–5.9 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} (Freeman, unpublished), which is comparable to the 0.1–1.3 x 10^6 eggs female^{-1} reported here. However, animal weights in the former study were approximately 25% heavier than here (see Table 8.7 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 higher 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 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 interval required to condition *H. australis* to oocyte maturation (determined histologically) was ≥1400 EATºC-days (using a BZP estimate of 5.0), similar to the optimal conditioning interval for spawning of female *H. rubra* and *H. laevigata* (this study) and *H. discus hannai* (Kikuchi and Uki, 1974a). Kabir (2001) also reported that the interval required to condition another New Zealand species, *H. iris*, was ≥2700 EATºC-days (using a BZP estimate of 6.2), similar to that for the optimal spawning response of *H. discus* (Kikuchi and Uki, 1974b).

The interval required for oogenesis of the tropical abalone, *H. asinina*, is short. This species is unusual in that spawning (which is seasonal, from October to April) is linked to a semilunar cycle and occurs every 13–15 days, but oogenesis requires more than two spawning cycles (i.e. 28–40 d) to complete, meaning there are at least two cohorts of oocytes in the ovary at any one time (Jebreen et al., 2000; Counihan et al., 2001). In most species the BZP for gonad development is the same as for larval development (Kikuchi and Uki, 1974a,b; Seki and Kanno, 1977; Kabir, 2001). Therefore, in *H. asinina*, using the BZP for larval development of 15ºC (Sawatpeera et al., 2001) as the estimated BZP for gonad development, the EAT for oogenesis at 26ºC (the mean seawater temperature during the spawning season; Counihan et al., 2001) can be calculated at 310–440 EATºC-days. 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 gonad maturation and spawning of abalone may relate to species differences in the activity and/or complexity of the biochemical pathways that control gonad development. Further studies on the spawning performance and hormonal control of reproduction in Haliotids relative to the EAT conditioning interval need to be undertaken to fully understand the reasons behind these differences.

8.5.2 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 et al. (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). This author also reported a significant decrease in mean spawning response times of *H. laevigata* when induced again after a four month conditioning period. This result was most probably due to the low spawning rate at the first induction rather than the animals becoming more responsive to inductions. Animals that did not spawn at the initial attempt would continue to mature, producing more eggs and being more likely to spawn early at the second induction.

Kikuchi and 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 and Uki (1974c).

Altering the time when induction commences, relative to the photoperiod, can also affect spawning response times in abalone. Uki and 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.

8.5.3 Gamete quality

Sperm motility, egg fertilization and larval hatchout rates were similar across both temperatures and all conditioning intervals. In all cases, they were high; generally, sperm motilities were >70%, while egg fertilization rates were >90%. Larval hatchout rates were typically only 5% less than fertilization rates. That egg viability is independent of conditioning interval is supported by Kikuchi and Uki (1974a, b) and Plant (2002). However, Plant et al. (2002) found that fertilization rate increased proportionally with conditioning time, peaking at 120 days.

8.6 Conclusions

Both the mean and total gamete production were higher for male and female *H. rubra* and *H. laevigata* cultured at 16ºC. The recommended duration of conditioning 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) and ≥1930 EATºC-days (212 days) for males and females, respectively. There appears to be considerable flexibility in the time that blacklip and greenlip abalone may be conditioned on artificial 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 fed an artificial diet. 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.

8.7 Acknowledgements

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Table 8.1. Spawning (% spawning) and repeat spawning (% repeat spawners) rate at successive inductions of female and male blacklip abalone relative to temperature (T°C) and conditioning interval (EAT). n = sample size, Mort = mortalities between inductions. Comparisons made within sex and within column. Numeric superscripts refer to statistic used. Alphabetic superscripts indicate location of significant ($P < 0.05$) differences.

				Induction 1		Induction 2	% repeat
Sex	T°C	EAT	n	% spawning[1]	Mort	% spawning[1]	spawners[2]
Female	16	1200	12	17	2	50	20
		1400	12	58	1	46	36
		1600	12	67	0	42	33
		1800	11	64	0	36	27
		2000	12	50	0	42	42
		mean ± sem		49 ± 4		43 ± 2	32 ± 2
	18	1200	15	67	5	70	40
		1400	13	46	3	70	30
		1600	10	60	0	100	60
		1800	12	83	2	100	80
		2000	12	58	0	58	42
		mean ± sem		63 ± 3		80 ± 9[a]	46 ± 9[b,c]
Male	16	1200	12	100	0	92	90
		1400	12	75	0	75	50
		1600	13	100	0	92	92
		1800	12	100	2	90	90
		2000	13	92	1	67	58
		mean ± sem		93 ± 5		83 ± 5	76 ± 9
	18	1200	11	100	1	100	100
		1400	12	100	0	100	100
		1600	12	100	0	100	100
		1800	11	100	1	80	90
		2000	12	83	0	92	83
		mean ± sem		97 ± 3		94 ± 4	93 ± 3[b,d]

1) One-way ANOVA
2) G-test

a) Mean spawning rate at 18°C > 16°C
b) % repeat spawners 18°C > 16°C
c) % non spawners 16°C > 18°C
d) % single spawners 16°C > 18°C

Table 8.2. Blacklip abalone response time, individual gamete production (Individual G.P.) and group mean gamete production relative to sex, induction number and temperature. Range refers to minimum and maximum values recorded across the five EAT conditioning groups. Optimal group is the group containing the maximum value of the factor, except in the case of response time, where the minimum value is given. Roman numerals refer to statistic used. Numeric superscripts refer to location of significant difference. Dash indicates no test, yes = $P < 0.05$, no = $P > 0.05$.

				Temperature									
				16°C				18°C					
Sex	Induction	Factor		Mean	(range)	Optimal group	EAT significance	Mean	(range)	Optimal group	EAT significance	Temperature significance	T*EAT interaction
Female	1	Response time[i,ii]	(h:min)	6:32	(5:53-7:10)	2000	no	6:05	(5:21-6:28)	1800	no	no	no
		Individual G.P.	(x 10^6)	1.3	(0.02-3.8)	1200	-	0.8	(0.05-3.9)	2000	-	-	-
		Group mean[i,ii]	(x 10^6)	1.3	(0.9-2.0)	1600	no	0.8	(0.5-1.5)	1600	no	no	no
	2	Response time[i,ii]	(h:min)	7:02	(6:43-7:12)	1600	no	6:27	(5:57-6:44)	1800	no	no	no
		Individual G.P.	(x 10^6)	1.1	(0.07-4.8)	1400	-	0.6	(0.01-4.4)	1200	-	-	-
		Group mean[i,ii]	(x 10^6)	1.1	(0.3-2.1)	1400	yes[1]	0.6	(0.2-11.1)	1200	yes	yes[3]	yes[4]
		IndDiff	(x 10^6)	3.4	(2.7-4.1)	1400	no	3.6	(3.0-4.4)	1200	no	no	no
		IndTotal	(x 10^6)	1.7	(1.2-2.2)	1400	no	1.1	(0.7-1.6)	1200	no	yes[3]	no
Male	1	Response time[i,ii]	(h:min)	6:15	(5:23-7:25)	1200	no	5:45	(5:33-6:05)	1800	no	no	yes[4]
		Individual G.P.	(x 10^{11})	3.7	(0.02-9.9)	2000	-	2.4	(0.01-11.1)	1800	-	-	-
		Group mean[i,ii]	(x 10^{11})	3.7	(1.1-6.0)	1800	yes[5]	2.4	(1.2-4.8)	1800	no	yes[3]	no
	2	Response time[i,ii]	(h:min)	6:41	(5:33-7:27)	1800	no	6:24	(5:38-7.26)	2000	no	no	yes[4]
		Individual G.P.	(x 10^{11})	3.6	(0.05-9.8)	2000	-	2.1	(0.04-10.5)	2000	-	-	-
		Group mean[i,ii]	(x 10^{11})	3.6	(1.5-5.3)	1600	yes[6]	2.1	(1.2-5.9)	2000	yes[7]	yes[3]	yes[4]
		IndDiff	(x 10^{11})	9.5	(8.2-11.1)	1400	no	9.5	(6.1-13.9)	2000	yes	no	yes[8]
		IndTotal	(x 10^{11})	7.3	(2.7-10.2)	1600	yes[9]	4.6	(2.6-8.4)	2000	no	yes[3]	yes[9]

i) One-way ANOVA
ii) Two-way ANOVA (T*EAT)

1) $1200^a\ 1400^a\ 1800^a\ 2000^a\ 1600^b$
2) $1200^a\ 1400^b\ 1600^b\ 1800^b\ 2000^b$
3) 16°C > 18°C
4) No discernible trend
5) $1800^a\ 1600^{ab}\ 2000^{abc}\ 1400^{bcd}\ 1200^d$
6) $1600^a\ 1400^{ab}\ 2000^{ab}\ 1800^{ab}\ 1200^b$
7) $2000^a\ 1200^b\ 1600^b\ 1400^b\ 1800^b$
8) 2000 @ 18°C > all others
9) Increase proportional to EAT interval

Table 8.3. Spawning (% spawning) and repeat spawning (% repeat spawners) rate at successive inductions of female and male greenlip abalone relative to temperature (T°C) and conditioning interval (EAT). n = sample size, Mort = mortalities between inductions. Comparisons made within sex and within column. Numeric superscripts refer to statistic used. Alphabetic superscripts indicate location of significant ($P < 0.05$) differences.

				Induction 1		Induction 2	% repeat
Sex	T°C	EAT	n	% spawning[1]	Mort	% spawning[1]	spawners[2]
Female	16	1200	13	31	1	17	8
		1400	11	83	0	73	63
		1600	12	33	2	100	30
		1800	10	60	0	75	60
		2000	12	75	0	75	75
		mean ± sem		56 ± 11		68 ± 14	47 ± 2[a,b]
	18	1200	12	25	1	36	9
		1400	12	50	0	33	25
		1600	12	25	2	70	10
		1800	11	45	0	100	45
		2000	12	42	2	80	50
		mean ± sem		37 ± 5		64 ± 13	28 ± 9
Male	16	1200	12	55	3	89	33
		1400	11	91	1	80	70
		1600	11	100	2	78	66
		1800	12	58	2	80	55
		2000	12	83	0	92	75
		mean ± sem		77 ± 9		84 ± 3	60 ± 7
	18	1200	12	25	2	80	20
		1400	12	25	1	100	18
		1600	12	75	0	100	75
		1800	11	27	2	78	11
		2000	12	58	2	80	40
		mean ± sem		42 ± 10[c]		88 ± 5	31 ± 12[d,e]

1) One-way ANOVA
2) G-test

a) % non spawners in 1200 group @ 16°C > all others at 16°C
b) % repeat spawners 1200 group @ 16°C < all others at 16°C
c) Mean spawning rate at 16°C > 18°C
d) % single spawners in 1200 & 1400 groups @ 18°C > all others @ 18°C
e) % repeat spawners in 1600 group @ 18°C > all others @ 18°C

Table 8.4. Greenlip abalone response time, individual gamete production (Individual G.P.) and group mean gamete production relative to sex, induction number and temperature. Range refers to minimum and maximum values recorded across the five EAT conditioning groups. Optimal group is the group containing the maximum value of the factor, except in the case of response time, where the minimum value is given. Roman numerals refer to statistic used. Numeric superscripts refer to location of significant difference. Dash indicates no test, yes = $P < 0.05$, no = $P > 0.05$.

				Temperature									
				16°C				18°C					
Sex	Induction	Factor		Mean	(range)	Optimal group	EAT significance	Mean	(range)	Optimal group	EAT significance	Temperature significance	T*EAT interaction
Female	1	Response time[i,ii]	(h:min)	8:50	(8:10-9:40)	1600	no	9:14	(8:50-9:47)	1200	no	no	no
		Individual G.P.	(x 10^6)	1.1	(0.2-3.5)	2000	-	0.4	(0.02-1.7)	1400	-	-	-
		Group mean[i,ii]	(x 10^6)	1.1	(0.4-1.7)	2000	yes[1]	0.4	(0.1-0.7)	1200	no	yes[2]	no
	2	Response time[i,ii]	(h:min)	9:15	(9:01-9:35)	1600	no	8:56	(8:32-9:20)	1400	no	no	no
		Individual G.P.	(x 10^6)	1.6	(0.1-5.9)	1800	-	1.0	(0.03-2.5)	1800	-	-	-
		Group mean[i,ii]	(x 10^6)	1.6	(1.3-1.9)	1800	no	1.0	(0.7-1.6)	1200	no	yes[2]	no
		IndDiff	(x 10^6)	3.3	(2.6-4.0)	1800	no	3.5	(3.0-4.2)	2000	no	no	no
		IndTotal	(x 10^6)	1.9	(1.4-2.5)	2000	no	1.03	(0.8-1.9)	2000	no	yes[2]	no
Male	1	Response time[i,ii]	(h:min)	9:24	(8:50-10:08)	1200	no	8:38	(8:25-9:15)	1800	no	no	no
		Individual G.P.	(x 10^{11})	0.5	(0.001-4.9)	1600	-	0.04	(0.002-0.2)	2000	-	-	-
		Group mean[i,ii]	(x 10^{11})	0.5	(0.006-1.0)	1600	yes[3]	0.04	(0.008-0.08)	2000	no	yes[2]	yes[4]
	2	Response time[i,ii]	(h:min)	8:09	(6:53-9:24)	2000	no	8:50	(8:19-9:12)	1600	no	no	yes[5]
		Individual G.P.	(x 10^{11})	1.9	(0.01-7.0)	1600	-	0.5	(0.008-3.2)	1600	-	-	-
		Group mean[i,ii]	(x 10^{11})	2.0	(0.6-2.7)	2000	no	0.5	(0.4-0.8)	1600	no	yes[2]	no
		IndDiff	(x 10^{11})	5.8	(5.1-6.6)	2000	no	5.1	(4.9-5.4)	1600	no	yes[2]	no
		IndTotal	(x 10^{11})	2.0	(0.4-3.3)	2000	yes[6]	0.5	(0.4-0.7)	1600	no	no	yes[7]

i) One-way ANOVA
ii) Two-way ANOVA (T*EAT)

1) 1600^a 2000^a 1200^a 1800^a 1400^b
2) 16°C > 18°C
3) 1600^a 2000^{ab} 1800^{abc} 1200^{cd} 1400^d
4) 1600 → 2000 @ 16°C > all others
5) No discernible trend
6) 1600^a 2000^{ab} 1800^{abc} 1200^{cd} 1400^d
7) Proportional increase with EAT @ 16°C

Table 8.5. Mean ± SEM sperm motility, egg fertilization and larval hatchout rates of blacklip (BL) and greenlip (GL) abalone relative to adult conditioning temperature and interval (EATºC-days). Only eggs from females spawning before 22:00 h were used. Dash indicates data not available. Sample size in parentheses.

Species	EATºC-	16ºC			18ºC		
	days	% Motility	% Fertilization	% Hatchout	% Motility	% Fertilization	% Hatchout
BL	1200	77 ± 5 (6)	82 ± 3 (3)	79 ± 3 (3)	79 ± 4 (7)	92 ± 2 (7)	88 ± 2 (7)
	1400	67 ± 8 (5)	98 ± 1 (2)	95 ± 0 (2)	92 ± 1 (8)	92 ± 1 (6)	80 ± 4 (6)
	1600	86 ± 3 (9)	89 ± 2 (2)	87 ± 1 (2)	87 ± 5 (10)	99 ± 0 (7)	98 ± 4 (7)
	1800	95 ± 0 (6)	-	-	72 ± 5 (6)	94 ± 3 (3)	92 ± 2 (3)
	2000	94 ± 1 (7)	98 ± 1 (2)	96 ± 1 (2)	92 ± 1 (11)	96 ± 2 (3)	93 ± 2 (3)
GL	1200	77 ± 7 (5)	83 ± 8 (2)	76 ± 9 (2)	67 ± 8 (6)	91 ± 2 (3)	73 ± 9 (3)
	1400	71 ± 4 (7)	92 ± 3 (4)	90 ± 3 (4)	74 ± 9 (4)	94 ± 1 (4)	79 ± 3 (4)
	1600	82 ± 4 (5)	91 ± 3 (3)	87 ± 3 (3)	85 ± 3 (5)	92 ± 2 (4)	92 ± 2 (4)
	1800	93 ± 1 (10)	95 ± 1 (4)	91 ± 1 (4)	78 ± 6 (4)	87 ± 3 (2)	83 ± 3 (2)
	2000	89 ± 8 (11)	96 ± 1 (2)	90 ± 1 (2)	86 ± 4 (4)	93 ± 2 (5)	90 ± 2 (5)

Table 8.6. Estimated EAT, based on a BZP of 7.5°C, and true EAT for blacklip and greenlip abalone, based on BZP values of 7.8°C and 6.9°C, respectively (Grubert and Ritar, in prep). True EAT is calculated using a water temperature of 16°C.

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

Table 8.7. Instantaneous fecundity 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 diet; P. c. = *Phyllospora comosa*; N. l. = *Nereocystis luetkeana*; P. m. = *Palmaria mollis*.

Species	Instantaneous fecundity (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 et al. (2001)
	137	±	1					G. b. and artificial	
	126	±	2					Artificial	
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 et al. (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 and Keesing (1999)
	100	–	1 400	107	–	142	CWC	Artificial*	Plant (2002)
	100	–	1 500+	88	–	142	CWC	Artificial*	K. Freeman, pers. comm.
	15	–	5 900	100	–	120	CWC	Artificial*	This study
H. rubra	1 910	±	290	100	–	125	CWC	Artificial*	Savva et al. (2000)
	1 710	±	570					P. c. and artificial*	
	1 110	±	300					P. c.	
	54	–	5 900	120	–	142	WC	–	Litaay and De Silva (2001)
	20	–	2 600	109	±	1	CWC	Artificial*	Plant et al. (2002)
	15	–	4 800	100	–	130	CWC	Artificial*	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 et al. (1998)
	181	±	133	89	±	5	Cult.	P. m.	
H. tuberculata	20	–	1 600		–		WC	–	Clavier (1992)

8.9 Addendum: Conditioning broodstock at shorter intervals

The investigation on "The effect of temperature and conditioning interval on the spawning success of wild-caught blacklip (*Haliotis rubra*) and greenlip (*H. laevigata*) abalone fed an artificial diet" revealed inconsistencies in the spawning at the first and second inductions of animals conditioned for repeated intervals of 1200 to 2000 EAT°C-days. It was suspected that this may have been due to spontaneous spawnings, particularly with the longer conditioning intervals of 1800 and 2000 EAT°C-days. A follow-up investigation was therefore undertaken into the effect of shorter conditioning intervals (i.e. <1200 EAT°C-days) on spawning success with the intention of reducing the frequency of spontaneous spawnings and thus increasing gamete yields after induction. Animals of each species and sex from the preceding five conditioning treatments (1200, 1400, 1600, 1800, 2000 EAT°C-days) at 16 or 18°C were re-allocated into three new treatments of 800, 1200 and 1600 EAT°C-days at each temperature. Within the limited timeframe, the 800 groups were conditioned and induced to spawn three times and the 1200 and 1600 groups were induced twice.

The proportion of blacklip females that spawned ranged between 17-69% at 16°C and 11-67% at 18°C (Table 8.8), while the proportions for males that spawned was higher and ranged between 36-100% at 16°C and 90-100% at 18°C (Table 8.9). The proportion of spawning greenlip females were between 13-100% at 16°C and 0-87% at 18°C, while the proportions for spawning males was generally higher, between 40-100% at 16°C and 33-100% at 18°C. Mean (for individuals) and total (for groups of animals) egg production in blacklip females was variable and there appeared to be no clear relationship with temperature or interval, although there appeared to be an overall tendency for better spawning performance at 16°C than at 18°C. This was also the case for greenlip females. No such trend was apparent for sperm production, with similar spawning performance of males at 16°C and 18°C and no difference between conditioning intervals.

These results are instructive even though there was no apparent difference between treatments for either the proportion of animals spawning, repeat spawning or gamete production. It was evident that there was high variability between treatment groups in responsiveness; occasionally no animals could be induced to spawn. On other occasions, there were much reduced numbers of gametes produced. In addition, repeated conditioning at the shortest intervals may be insufficient to allow maturation of all gametes. Hence, only those gametes that were mature when induced were spawned, which could partly explain some of the variability in the data.

The overall spawning performance was lower in this experiment, where animals were conditioned over 2-3 intervals, compared to the preceding experiment, where animals had already been conditioned for 2 intervals. The lower performance may be attributed to the long duration for which the animals were held (up to 5 conditioning intervals and >2 years) resulting in depletion of some body reserves utilised in gamete production. It appears that there may have been an insufficient supply of essential components from the diet to replenish these reserves. However, such speculation needs to be followed through with a long-term study on the incorporation and depletion of body components supplied by the diet, most likely related to lipids, for gamete production.

Table 8.8. Spawning performance of blacklip females and males induced repeatedly after conditioning for 800, 1200 or 1600 EAT°C-days at 16 or 18°C.

EAT interval	Induction number		Females held at 16°C	Females held at 18°C		Males held at 16°C	Males held at 18°C
800	1	Mean eggs±sem ($x10^6$)	0.86 ± 0.56	0.54 ± 0.21	Mean sperm±sem ($x10^9$)	188 ± 5	101 ± 23
(94 days @ 16°C,		Total eggs ($x10^6$) $group^{-1}$	5.13	3.23	Total sperm ($x10^9$) $group^{-1}$	2260	1110
76 days @ 18°C)		% spawned, n	46% 13	67% 9	% spawned, n	86% 14	100% 11
	2	Mean eggs±sem ($x10^6$)	1.34 ± 0.24	0.39 ± 0.00	Mean sperm±sem ($x10^9$)	521 ± 132	397 ± 82
		Total eggs ($x10^6$) $group^{-1}$	9.42	0.39	Total sperm ($x10^9$) $group^{-1}$	2600	3970
		% spawned, n	54% 13	11% 9	% spawned, n	36% 14	91% 11
	3	Mean eggs±sem ($x10^6$)	0.92 ± 0.69	1.26 ± 0.35	Mean sperm±sem ($x10^9$)	278 ± 96	300 ± 89
		Total eggs ($x10^6$) $group^{-1}$	1.84	7.57	Total sperm ($x10^9$) $group^{-1}$	2780	3000
		% spawned, n	17% 12	67% 9	% spawned, n	100% 10	91% 11
1200	1	Mean eggs±sem ($x10^6$)	1.63 ± 0.46	0.64 ± 0.13	Mean sperm±sem ($x10^9$)	34 ± 8	325 ± 91
(141 days @ 16°C,		Total eggs ($x10^6$) $group^{-1}$	6.53	2.58	Total sperm ($x10^9$) $group^{-1}$	101	3900
114 days @ 18°C)		% spawned, n	40% 10	27% 15	% spawned, n	30% 10	92% 12
	2	Mean eggs±sem ($x10^6$)	1.18 ± 0.38	0.91 ± 0.12	Mean sperm±sem ($x10^9$)	72 ± 25	107 ± 29
		Total eggs ($x10^6$) $group^{-1}$	5.89	0.12	Total sperm ($x10^9$) $group^{-1}$	717	963
		% spawned, n	50% 10	50% 10	% spawned, n	90% 10	90% 10
1600	1	Mean eggs±sem ($x10^6$)	0.87 ± 0.25	1.24 ± 0.56	Mean sperm±sem ($x10^9$)	135 ± 67	312 ± 58
(188 days @ 16°C,		Total eggs ($x10^6$) $group^{-1}$	7.87	7.43	Total sperm ($x10^9$) $group^{-1}$	677	6750
152 days @ 18°C)		% spawned, n	69% 13	50% 12	% spawned, n	39% 13	100% 12
	2	Mean eggs±sem ($x10^6$)	1.69 ± 0.39	1.49 ± 0.09	Mean sperm±sem ($x10^9$)	257 ± 75	289 ± 66
		Total eggs ($x10^6$) $group^{-1}$	3.39	2.98	Total sperm ($x10^9$) $group^{-1}$	2052	2312
		% spawned, n	20% 10	20% 10	% spawned, n	80% 10	89% 9

Table 8.9. Spawning performance of greenlip females and males induced repeatedly after conditioning for 800, 1200 or 1600 EAT°C-days at 16 or 18°C.

EAT interval	Induction number		Females held at 16°C	Females held at 18°C		Males held at 16°C	Males held at 18°C
800	1	Mean eggs±sem ($x10^6$)	2.21 ± 0.26	0.14 ± -	Mean sperm±sem ($x10^9$)	61 ± 14	20 ± 8
(94 days @ 16°C,		Total eggs ($x10^6$) group^{-1}	13.20	0.14	Total sperm ($x10^9$) group^{-1}	605	159
76 days @ 18°C)		% spawned, n	43% 14	8% 13	% spawned, n	100% 10	67% 12
	2	Mean eggs±sem ($x10^6$)	1.13 ± 0.26	1.17 ± -	Mean sperm±sem ($x10^9$)	172 ± 48	4 ± 2
		Total eggs ($x10^6$) group^{-1}	13.50	1.17	Total sperm ($x10^9$) group^{-1}	1380	17
		% spawned, n	100% 12	10% 10	% spawned, n	100% 8	33% 12
	3	Mean eggs±sem ($x10^6$)	0.16 ± 0.04	0.91 ± 0.26	Mean sperm±sem ($x10^9$)	12 ± 4	94 ± 24
		Total eggs ($x10^6$) group^{-1}	0.93	7.31	Total sperm ($x10^9$) group^{-1}	47	1030
		% spawned, n	50% 12	80% 10	% spawned, n	50% 8	92% 12
1200	1	Mean eggs±sem ($x10^6$)	1.68 ± 0.42	1.22 ± 0.14	Mean sperm±sem ($x10^9$)	80 ± 23	95 ± 21
(141 days @ 16°C,		Total eggs ($x10^6$) group^{-1}	15.19	15.80	Total sperm ($x10^9$) group^{-1}	799	1520
114 days @ 18°C)		% spawned, n	75% 12	87% 16	% spawned, n	83% 12	100% 16
	2	Mean eggs±sem ($x10^6$)	1.68 ± 0.47	0.53 ± 0.21	Mean sperm±sem ($x10^9$)	88 ± 25	9 ± 5
		Total eggs ($x10^6$) group^{-1}	13.40	0.26	Total sperm ($x10^9$) group^{-1}	789	53
		% spawned, n	67% 12	42% 12	% spawned, n	75% 12	50% 12
1600	1	Mean eggs±sem ($x10^6$)	1.25 ± 0.37	0.39 ± 0.13	Mean sperm±sem ($x10^9$)	8 ± 3	54 ± 15
(188 days @ 16°C,		Total eggs ($x10^6$) group^{-1}	3.74	1.97	Total sperm ($x10^9$) group^{-1}	32	433
152 days @ 18°C)		% spawned, n	30% 10	42% 12	% spawned, n	40% 10	67% 12
	2	Mean eggs±sem ($x10^6$)	1.79 ± -	0 ± 0	Mean sperm±sem ($x10^9$)	52 ± 20	112 ± 37
		Total eggs ($x10^6$) group^{-1}	1.79	0	Total sperm ($x10^9$) group^{-1}	262	561
		% spawned, n	13% 8	0% 10	% spawned, n	50% 10	50% 10

9 ABALONE BROODSTOCK CONDITIONING SYSTEM AT TAFI MRL

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9.1 Introduction

There are many different variables to consider when building an abalone broodstock conditioning system. This article describes the system built for the FRDC Abalone Aquaculture Subprogram project "Commercial control of spawning in temperate abalone" at the Tasmanian Aquaculture & Fisheries Institute, Marine Research Laboratories (TAFI MRL). It also details systems commonly used by other researchers and farmers. It is intended as a guide only, with the ultimate decision on which configuration to adopt depending on things like space, installation and running costs. It must be noted that the system about to be described was built for experimental purposes and as such, not all features will be applicable to commercial situations.

9.2 The system at Tasmanian Aquaculture & Fisheries Institute, Marine Research Laboratories

9.2.1 Broodstock system:
The abalone broodstock conditioning system at TAFI MRL is located in an insulated room and consists of 32 x 150 L round, fibreglass tanks in four rows of eight tanks. The internal diameter of each tank is 800 mm and the walls are 300 mm deep. The drop from the base of the wall to the central, internal standpipe (40mm PVC pressure pipe) is 50 mm. The inner surface of each tank is black gel coat with white on the outer surface.

In order to determine the Biological Zero Point for gonad development of blacklip and greenlip abalone (part of the FRDC project), it was necessary to maintain them at 12, 14, 16 and 18°C. Therefore, each row of eight tanks had independent temperature control (set at the stated temperatures) achieved using a Carrier reverse cycle pool and spa heater (10kW heating, 6kW cooling), also known as a heat chill unit (Fig. 9.1). Each row has a sump and header tank of 110 and 320 L, respectively.

The seawater to the abalone room is filtered through 2 x 200 μm bag filters in parallel (Fig. 9.2). Water to each sump is supplied, and level maintained, by a 25 mm trough valve. It is then drawn out of the sump by an Onga 413 pump, passes through the heat chill and into the header tank (Fig. 9.3). As water is pumped to the header tank much faster than it drains to the broodstock tanks, the majority of the water returns to the sump via an overflow pipe from the header (Fig. 9.4). The supply lines to the broodstock tanks and header overflows are 50mm PVC pressure pipe while the pipe work in the heat chill circuit is 40 mm PVC pressure. Using several adaptors, the final supply line to each tank is 19mm black poly pipe (Fig. 9.5).

The flow rate to each 150L broodstock tank is set at approximately 2L/min (one exchange every 75 minutes) with water level maintained by an internal standpipe. An oversize sleeve with two slots at its base sits over the standpipe and draws wastes from the bottom of the tank. The sleeve also allows vigorous aeration without the loss of "new" water entering the tank. Water is not recovered once it leaves the broodstock tanks and flows to a drain.

Aeration to each tank is provided by a 30 cm air bath sourced from an aquarium supplier. Photoperiod is maintained at 12L:12D (light 0600h to 1800h) by 6 x 36W fluorescent tubes each covered by one layer of shade cloth. Light intensity at the bottom the broodstock tanks (when filled) is 90-100 Lux. Abalone are fed daily (1630h) on 3mm Adam & Amos broodstock chip and tanks siphoned every second day. Aeration is turned off while siphoning in order to let solids settle and make it easier to see waste products.

9.2.2 Spawning induction system:
The spawning induction system at TAFI MRL comprises 20 x 10L plastic tubs, two cartridge filters (50 and 1 μm nominal) an 110L header tank with a 2kW immersion heater, and 2 x 150W UV units (Australian Ultraviolet Manufacturing, NSW - Fig. 9.6). Water is drawn from the header tank of the broodstock system from which the abalone were removed (to avoid temperature shock) and pumped through the cartridge filters by means of a small submersible pump. Pipe work is 19mm black poly. Water level in the spawning header tank is maintained by an overflow. Upon exiting the spawning header tank, water passes through the two UV units (plumbed in parallel) into each of the ten upper spawning tubs, with flow regulated at 300ml/min by means of a 13 mm valve. It then drains from the upper tub via a 13 mm black poly standpipe into the tub below (from which it later exists by the same means).

The night before the induction is to be conducted, a timer on the power supply to the submersible pump is set so that all spawning tubs will be filled by 0900h the next day. At that time, selected animals are placed in the spawning tubs (females in the upper and males in the lower tubs). Both UV units are then turned on, as is the immersion heater (set to 3°C above the conditioning temperature). Water in the header tank is mixed by vigorous aeration. Water temperature in the spawning tubs increases by 1°C/h for 3h after which time the heater is turned off. About six hours after the UV is turned on the males begin to spawn, followed by the females.

9.3 Points to consider

9.3.1 Temperature control - flow-through versus recirculation systems
Abalone broodstock conditioning requires stable temperatures, generally around 16-18°C. Several manufacturers produce heater chillers capable of maintaining water temperatures at ±0.3°C of the set point. The heating/cooling capabilities of a particular unit will dictate both the number of, and flow rate to, the broodstock tanks.

As mentioned, water only passes through our broodstock tanks once, rather than being recirculated. There are both advantages and disadvantages to this method. Perhaps the biggest advantage of using flow-through is that of water quality, as there is minimal build up of nitrogenous wastes, oxygen saturation is kept quite high (>90%) and it eliminates the need for biological filtration. A further advantage is that it reduces (but does not stop) spontaneous

spawning of broodstock. If eggs or sperm are released in one tank in a recirculating system, then this has the potential to trigger spawning of animals in adjacent tanks. In a flow-through system, the spawning event would at least be confined to just the one tank.

A major disadvantage of temperature controlled flow-through systems is that of heating/cooling costs. Recirculation systems are able to recover much of the energy used to heat or cool the water whereas flow-through systems are not (unless they are very sophisticated). Therefore, the cost of running a recirculating system is much less. Flow through systems may also require auxiliary heaters/coolers during periods of extreme high or low water (and/or air) temperatures. During winter, when the ambient water temperature at Taroona falls to 9°C, it has been necessary to add immersion heaters (2kW and 9kW) to the header tanks for the 16 and 18°C systems.

Ideally, air temperature in the broodstock room will also be controlled as this will aid in stabilising water temperature. Therefore, the broodstock room should be insulated; preferably with Bondor panelling and an air conditioner fitted and set to the same (or slightly higher) temperature as the conditioning system.

9.3.2 Alarm systems

It is most important to have temperature alarms on broodstock conditioning systems as large variations in water temperature can have serious effects on the conditioning process and also lead to stock loss. Flow alarms are also important, particularly in the case of pump failure, and could mean the difference between just repairing a pump and replacing it completely.

The heat chills we use all have high and low temperature alarm settings, and can be connected to an alarm system. Given the value of the abalone stock, it is assumed that most farms would have an alarm system fitted, and so the price of connecting inputs from the heat chills to an existing system would not be great. While our heat chills do have an inbuilt flow sensor (that turns the unit off if there is no water flow) this does not activate an alarm (at least in standard form). However, after discussing this feature with the suppliers of the unit, they were able to retro fit a relay from the flow sensor that activates an alarm when there is no flow.

9.3.3 Filtration

Filtration for the abalone broodstock conditioning system at TAFI MRL is rudimentary. Water supplied to the room passes through a large mesh screen (at the inlet) and 2 x 200μm bag filters. As the inlet is above a rocky reef, turbidity is low (except in times of prolonged rough weather). The bags are normally cleaned every second day but during periods of rough weather are cleaned morning and afternoon. This system works but is not ideal. Sand or drum filters would be preferred and recommended when building a broodstock system.

Filtration to the spawning induction system should be to 1μm (nominal) as particles flowing through the UV unit shadow the water behind them, reducing the effectiveness of the lamp. Particulate organic matter will also compromise the quality of eggs and sperm.

9.3.4 Sump and header configuration
Limited space in our broodstock conditioning room necessitated the use of small sumps and header tanks. For those people considering installing a temperature controlled flow-through system it would be possible to use one large (i.e. 2000 L) tank that acts as both a sump and a header tank. As long as the water level and feed line from this tank was above the water level in the broodstock tanks then there would be sufficient pressure to feed these tanks. Of course, the higher the tank and outlet, the greater the available pressure to the broodstock tanks.

9.3.4 Broodstock tank size
Choice of broodstock tank size largely depends on the users preference when it comes to spawning broodstock. One method is to use small (30 L) round plastic tubs stocked with three or six animals. The water supply to the broodstock tanks includes a feed line from a UV unit. When conducting a spawning run, water is diverted through the active UV chamber and flows into the broodstock tanks, inducing them to spawn.

Advantages of this system include that it occupies less space and eliminates potential handling stresses of moving animals to dedicated spawning tanks. However, if several of the animals in the tank spawn, it is difficult to determine the fecundity/ sperm production of each individual.

At TAFI MRL, we stock twenty five to thirty 100-120 mm in our 150L tanks. Undersized, mature animals are used rather than legal sized animals so as into increase replication without a large increase in biomass. An appropriate stocking density for legal size animals in our tanks would be 15-20 animals. Prior to a spawning run, abalone are removed from the broodstock tanks, visually inspected and those with ripe gonads placed individually into 10L plastic spawning tubs. The use of plastic tubs is not universal, as some people use glass aquaria, but we prefer the tubs because they are cheap, light and virtually unbreakable. Spawning individuals in separate tubs allows their performance to be recorded, enabling selective breeding of those animals that produce higher quality eggs and sperm.

9.3.5 Animal retention
As many abalone farmers know, blacklip abalone have a remarkable ability to escape from tanks, especially when they become gravid. They instinctively crawl to the highest point in the tank as they get ready to spawn. However, this high point (the edge of the tank) may soon be followed by a very low point (the floor of the conditioning room). We have devised a mesh abalone “fence” which has retained all animals thus far (Fig. 9.7). It consists of a ring of 19mm black poly pipe with the same outer diameter as the inner diameter of the broodstock tank, around which is folded 150mm wide “gutter guard”. The two sides of the gutter guard are then cable tied together at intervals of about 150mm producing a corrugated pattern on the inside of the ring. Cable ties are also used to secure the gutter guard to the ring at six or eight points around its circumference. Plastic upholstery fasteners (tent hooks) are then used to secure the abalone fence to the tank. The stud on the fasteners goes through the mesh while the hook slides over the edge of the tank.

9.3.6 Costs
Item costs given below were at December 2000 prices and do not include GST. If buying a single heat chill one can expect to pay more than the price quoted here as we bought four units. Prices are not given for UV sterilisation units as we salvaged existing units from the TAFI MRL

aquarium facility. It must be emphasised that this system was built to cope with an annual water temperature range of 9-20°C. In places with much higher minimum temperatures, auxiliary heaters may not be necessary (but there may be a need for greater chilling capacity).

9.4 Conclusion

The broodstock conditioning system described here is in some ways more sophisticated than would be necessary for commercial situations. For example, only one heat chill would be required in most instances. By contrast, our filtration system is less than ideal and we recommend sand or drum filtration. It may also not be commercially feasible to run a temperature controlled flow through system such as ours (at least in its present form). However, this system could be made more efficient by pumping waste water from the broodstock tanks through a heat exchanger located in the sump, thus recovering some of the energy used to heat the water.

Table 1. Costs of items used in the construction of abalone broodstock conditioning system

Item	Quantity	Unit Price	Total
Carrier heat chills [1]	4	$3700	$14800
Broodstock tanks[2]	32	$100	$3200
FSI bag filter housings[3]	2	$680	$1360
PVC pipe and fittings[4]			$2500
Philmac trough valves[5]	4	$40	$160
Sumps[6]	4	$85	$340
Header tanks[7]	4	$110	$440
Header stand*[8]			$200
Heat chill and water pump stand*[8]			$300
Tank stands*[9]			$200
Onga 413 water pumps[10]	4	$165	$660
2 kW immersion heater[11]			$485
2 kW spawning heater[11]			$760
9 kW immersion heater[12]			$1400
Spawning tubs[6]	20	$6	$120
Total			$26,925

1) Airco, Derwent Park, TAS;
2) Y-not Fibreglass, Launceston;
3) Australian Filter Specialists, Heidelberg Heights, VIC;
4) West Hobart Plumbing Centre, Hobart;
5) Websters, Hobart;
6) Associated Plastics, Cambridge, TAS;
7) Chris Ikin, Launceston;
8) Blackwoods Steel, Derwent Park, TAS;
9) K&D Warehouse, Hobart;
10) J.R. Stephenson, Hobart;
11) Austin & Cridland, Carlton, NSW;
12) Istra, Caringbah, NSW.

Items marked * are for materials only and do not include labour costs for construction.

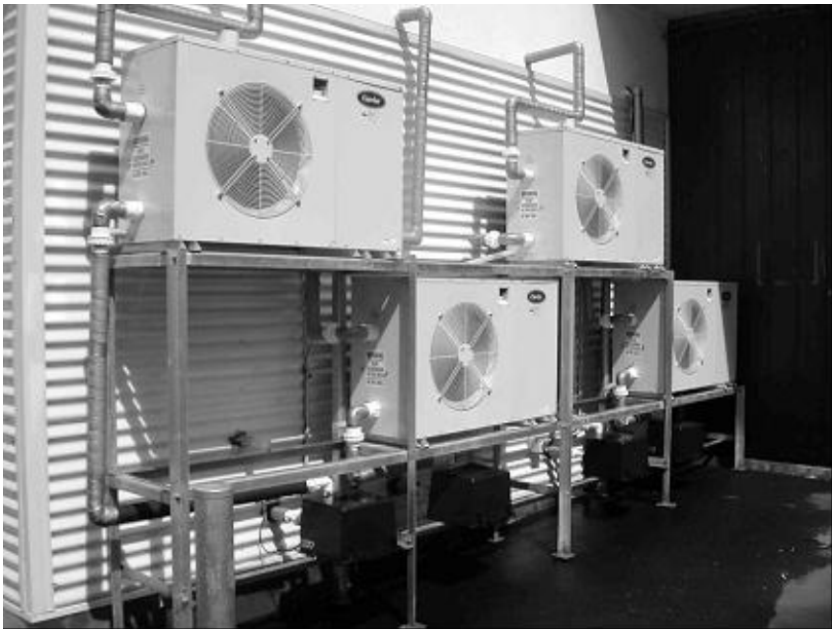

Fig. 9.1. Heat chill units set at 12, 14, 16 & 18°C supplying separate lines of broodstock tanks

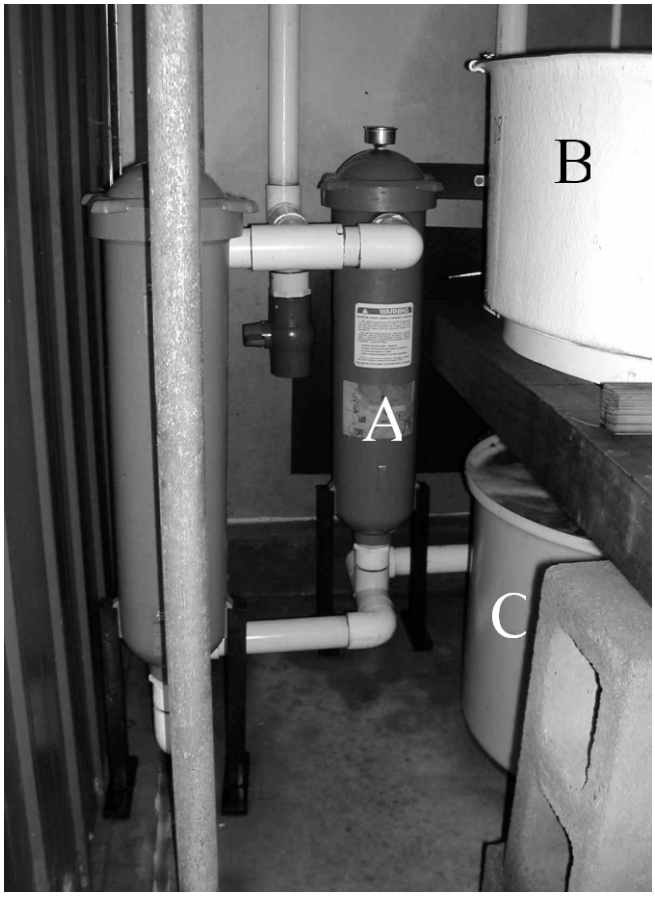

Fig. 9.2. Bag filter housings (A), broodstock tank (B) and sump (C).

Fig. 9.3. Header (A) and broodstock (B) tanks.

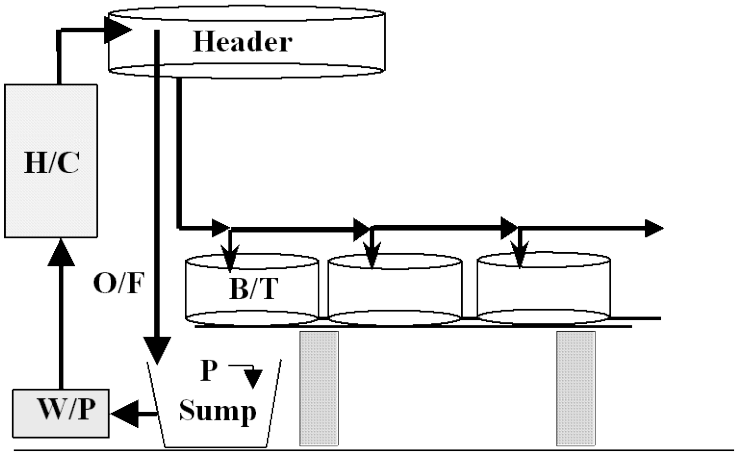

Fig. 9.4. Diagrammatic representation of flow through conditioning unit. P = Philmac trough valve; Sump = 110L tub; W/P = Water pump (0.4 kW); H/C = Heat chill (10kW heating, 6 kW cooling); Header = 320 L tank; O/F = Overflow; B/T = Broodstock tank.

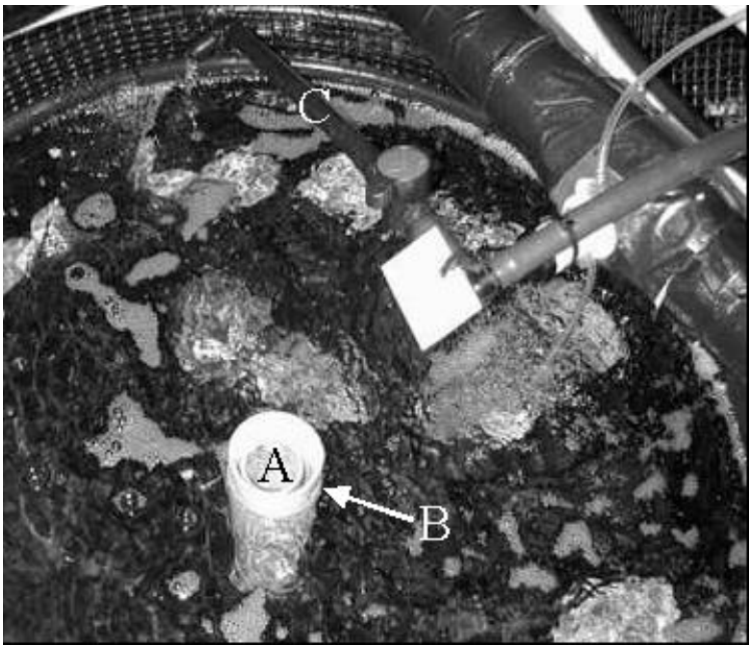

Fig. 9.5. Broodstock tank showing standpipe (A), sleeve (B) and 19mm supply line (C).

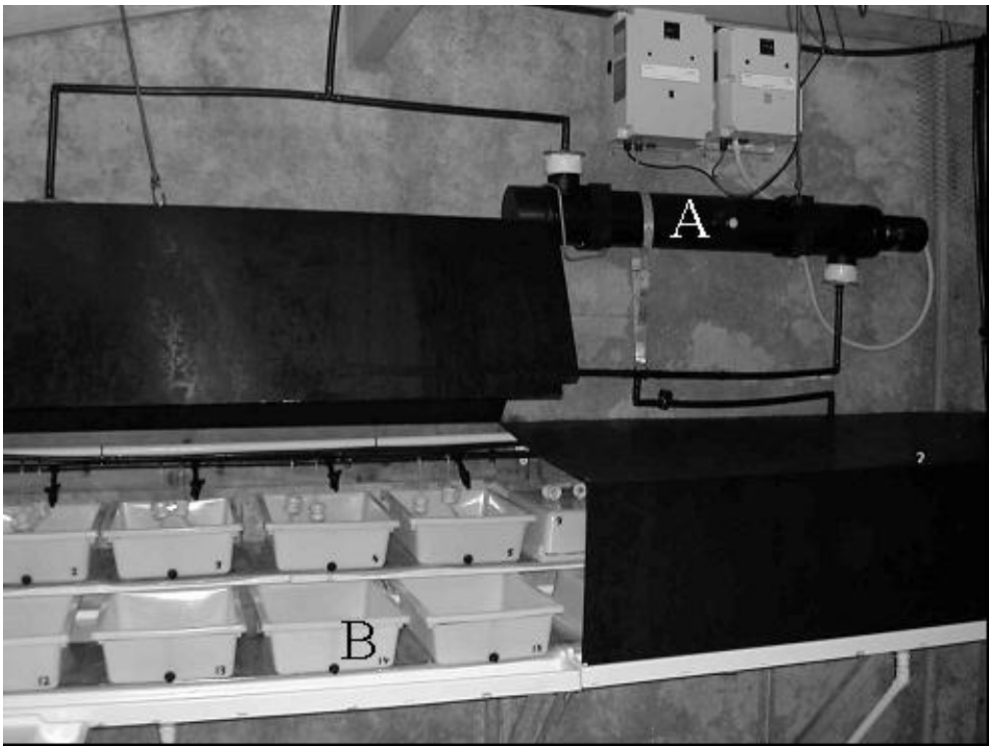

Fig. 9.6. UV water sterilisation units (A) and spawning tubs (B). Note - left hand UV unit obscured by cover.

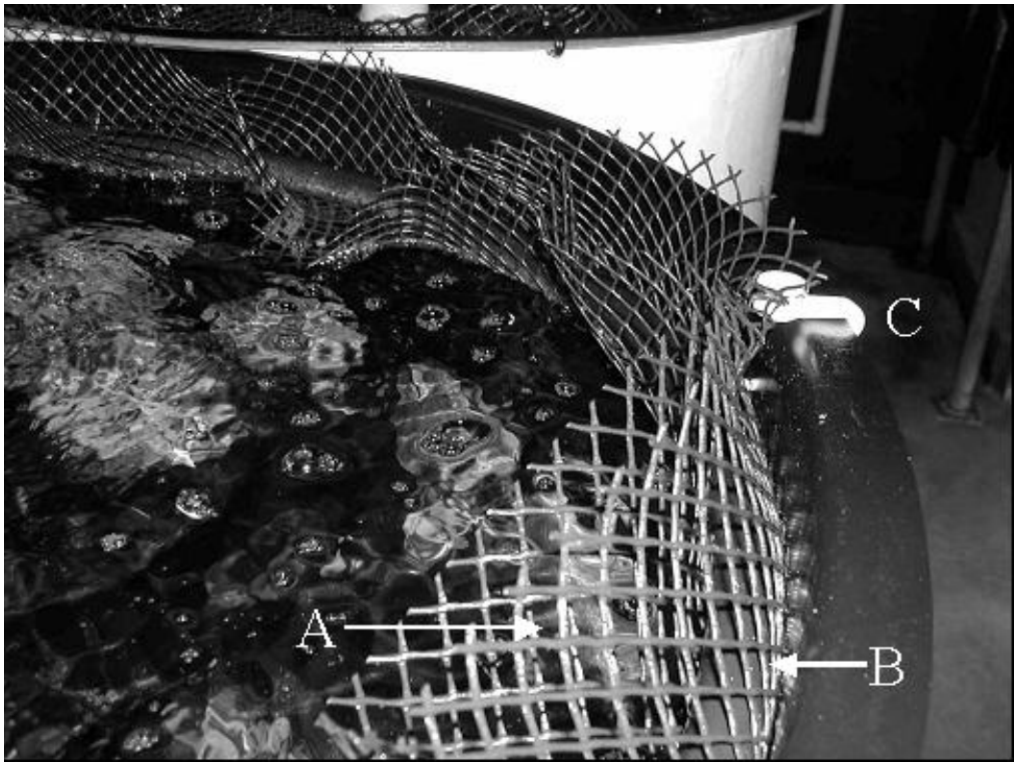

Fig. 9.7. Abalone “fence”, constructed using gutter guard (A), 19 mm black poly pipe (B) and tent hooks (C).

10 BENEFITS

The results of this study offer a detailed understanding of the reproductive biology of wild-caught blacklip and greenlip abalone conditioned in the hatchery at constant temperatures and on an artificial (or formulated) diet. This information will be most useful to commercial hatcheries desiring to provide seedstock not only during the normal spawning season but also out-of-season. Thus, there is a greater benefit to the entire abalone aquaculture sector. Some of the findings from this study have been incorporated in a how-to manual for the conditioning of abalone broodstock (to be published shortly) and this will be made available to hatcheries via the Abalone Aquaculture Subprogram.

The two species show many similar characteristics in their reproductive processes. Animals may be repeatedly conditioned and spawned yielding high quality eggs and sperm. Careful management of animals or groups of animals will allow them to be placed on cyclical regimes where they may be spawned repeatedly. The best results can be expected for animals conditioned at 16°C and, ideally, induced at intervals of 188 and 165 days for male and female blacklips, and 188 and 212 days for male and female greenlips. In practice, however, this may range between 4-8 months.

Although the nutritional requirements of abalone broodstock were not investigated, the formulated diet was sufficient to provide high quality gametes during the approximately two years under which animals were held. Further investigation on diet is warranted because it is likely that longer conditioning intervals or conditioning of broodstock animals that are still growing may place a greater requirement on some nutritional components.

The conditioning of animals requires considerable extra management and resources compared to the induction of animals recently caught from the wild and then discarded. For conditioning, animals need to be carefully maintained for many months in several tanks in a separate holding system with temperature control of the water. However, this allows animals to be observed and culled on the basis of poor development and growth, ensuring that only the best are used for induction.

The project benefits the abalone farming industry in southern Australia estimated as: South Australia 35%, Tasmania 20%, Victoria 35% and Western Australia 10%. These figures were determined by the Subprogram Leader in consultation with industry to reflect the relative size of the industry in 2000 and the expected development over the following 5 years based on current investment levels.

11 FURTHER DEVELOPMENT

The research determined that reproductive performance does not deteriorate over the first two conditioning cycles (up to 470 days). However, subsequent performance appears to result in a reduction in performance. This is most probably related to the conditioning environment of the abalone, more specifically to the diet. The diet used in this study was a modification of a typical grower diet. However, the physiological processes demanding mobilisation of some body components into gamete production may not have been met by the diet and this is an area worthy of further study.

This study examined animals from Tasmanian waters only, specifically from the north-east of the State. Although we have no evidence either way, the question remains "Is there an influence of environmental history on the BZP and thus on the optimum conditioning interval?" For example, do animals from South Australia or New South Wales have a different BZP to those from Tasmania? This could potentially implicate a genetic component in the conditioning interval. Both the environmental history and genetic profile of abalone may warrant further investigation to determine optimal conditioning regimes of animals obtained from other locations.

12 PLANNED OUTCOMES

The project outputs included:

- Considerable progress in understanding the reproductive biology of Australian temperate abalone.
- Discovery of information on the biological zero point (BZP) (the theoretical minimum temperature at which gonad growth and development begins) and the relationship between temperature and gonad development.
- Identification of the temperature required to condition abalone over a set period of time and to optimise the spawning of abalone.
- Development of practical procedures for the consistent and frequent spawning of abalone.
- Protocols developed for the commercial control of spawning. The protocols are applicable to industry users (abalone hatcheries) to reliably spawn selected animals as required, including wild-caught broodstock early in the spawning or out-of-season, and for the more efficient production of hybrid abalone. This, in turn, provides a predictable flow of seedstock to the growout sector of abalone farming.

These outputs have contributed to meet the planned outcomes, as identified in the original application, which were:

- Reduced risk of spawning failure
- Increased wealth generation through increased production
- Increased efficiency of production
- Decreased production time

The project benefits the abalone farming industry in Tasmania, Victoria, South Australia and Western Australia. The magnitude of the benefit outlined in B7 Flow of Benefits in the project application. Flow of Benefits was determined in 2000 by the Subprogram Leader in

consultation with industry to reflect the relative size of the industry in each state and the expected development over the following 5 years based on current investment levels.

13 CONCLUSIONS

The project successfully achieved its objectives, which were:

1. To determine the biological zero point (BZP) and the relationship between temperature and gonad development.
2. To identify the temperature required to condition abalone over a set period of time.
3. To develop protocols for the commercial control of spawning in abalone by temperature manipulation.

The principal conclusion from the research was that hatcheries for blacklip and greenlip abalone are using and will continue to use the results of the study to improve their broodstock conditioning. The results have led to considerable progress in understanding the reproductive biology of Australian temperate abalone and provided practical procedures for their consistent and frequent spawning. The project provided information on the BZP and the relationship between temperature and gonad development. It identified the temperature required to condition abalone over a set period of time and optimised the spawning of abalone. From this information, protocols were developed for the commercial control of spawning. The protocols are applicable to industry users (abalone hatcheries) to reliably spawn selected animals as required, including wild-caught broodstock early in the spawning or out-of-season, and for the more efficient production of hybrid abalone. This, in turn, provides a predictable flow of seedstock to the growout sector of abalone farming.

APPENDIX 1 - INTELLECTUAL PROPERTY

Any intellectual property generated through this project is protected based on the share of contributions outlined in C7 of the funding application. Given the ongoing nature of this research it is difficult to identify any specific intellectual property generated. Therefore, final distribution of the report should be considered carefully.

APPENDIX 2 - PROJECT STAFF

Staff that participated in this project are:

Dr Arthur Ritar (Senior Research Fellow – TAFI);

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Ms Debbie Gardner (Technical officer, TAFI)

Ms Jo Walker (Technical officer, TAFI)

APPENDIX 3 - RELATED PUBLICATIONS

Journal articles

Grubert, M.A. and Ritar, A.J. Temperature effects on the dynamics of gonad and oocyte development in captive wild-caught blacklip (*Haliotis rubra*) and greenlip (*H. laevgata*) abalone. Invertebrate Reproduction and Development (in press)

Grubert, M.A. and Ritar, A.J. The effect of temperature on the embryonic and larval development of blacklip (*Haliotis rubra*) and greenlip (*H. laevigata*) abalone. Invertebrate Reproduction and Development (in press)

Grubert, M.A. and Ritar, A.J. The effect of temperature and conditioning interval on the spawning success of wild-caught blacklip (*Haliotis rubra*) and greenlip (*H. laevigata*) abalone fed an artificial diet. Aquaculture Research (submitted)

Grubert, M.A., Dunstan, G.A. and Ritar, A.J. The effect of temperature and spawning status on the lipid and fatty acid composition of captive and wild-caught blacklip (*Haliotis rubra*) and greenlip (*H. laevigata*) abalone fed a formulated feed. Aquaculture (submitted)

Conference and other presentations

Ritar, A.J. (2000). Conditioning of greenlip and blacklip abalone by temperature manipulation: development of an experimental protocol. *Proc. 7th Annual Abalone Aquaculture Workshop*, Dunedin, New Zealand, 52-54.

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Grubert, M.A. and Ritar, A.J. (2002). Estimation of the biological zero point of blacklip (*Haliotis rubra*) and greenlip (*H. laevigata*) abalone. *Proceedings of the 9th Annual Abalone Aquaculture Workshop*, 29-31 August 2002, Queenscliff, Victoria, 63-75.

Ritar, A.J. and Grubert, M.A. (2002). Conditioning of wild-caught blacklip and greenlip abalone broodstock. *Proceedings of the 9th Annual Abalone Aquaculture Workshop*, Queenscliff, Victoria, 76-86.

Grubert, M.A., Ritar, A.J. and Mundy, C.N. (2003). Aspects of the fertilization biology of wild-caught blacklip (*Haliotis rubra*) and greenlip (*H. laevigata*) abalone conditioned on an artificial diet. In: *Proceedings of the 5th International Abalone Symposium*, 12-17 October 2003, Qingdao, China. 63.

Grubert, M.A., Elliott, N.G. and Ritar, A.J. (2003). Triploid induction of blacklip and greenlip abalone using water pressure and 6-Dimethylaminopurine (6-DMAP): Experimental protocol. In: *Proceedings of the 10th Annual Abalone Aquaculture Workshop*, 19-21 November 2003, Port Lincoln. Fleming, A.E. (Editor). Abalone Aquaculture Subprogram, Fisheries Research and Development Corporation, Australia. 1-3.

Grubert, M.A. and Ritar, A.J. (2003). The effect of temperature and conditioning interval on the spawning success of wild-caught blacklip (*Haliotis rubra*) and greenlip (*H. laevigata*) abalone fed an artificial diet. In: *Proceedings of the 10th Annual Abalone Aquaculture Workshop*, 19-21 November 2003, Port Lincoln. Fleming, A.E. (Editor).

Abalone Aquaculture Subprogram, Fisheries Research and Development Corporation, Australia. 5-35.

Grubert, M.A. and Ritar, A.J. (2003). Temperature effects on the dynamics of gonad and oocyte development in captive wild-caught blacklip (*Haliotis rubra*) and greenlip (*H. laevigata*) abalone. In: *Proceedings of the 10th Annual Abalone Aquaculture Workshop*, 19-21 November 2003, Port Lincoln. Fleming, A.E. (Editor). Abalone Aquaculture Subprogram, Fisheries Research and Development Corporation, Australia. 37-55.

Grubert, M.A. and Ritar, A.J. (2002). Abalone broodstock conditioning system at TAFI MRL. *Austasia Aquaculture* 16(3), 29-36.

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Ritar, A.J. (2001). Conditioning of wild abalone broodstock', *Ab-brief, Fisheries Research and Development Corporation*, West Deakin, ACT, Australia, 5, 1.

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APPENDIX 4 - THE EFFECT OF TEMPERATURE ON THE EMBRYONIC AND LARVAL DEVELOPMENT OF BLACKLIP (*HALIOTIS RUBRA*) AND GREENLIP (*H. LAEVIGATA*) ABALONE

Mark A. Grubert and Arthur J. Ritar

This chapter published as:
Grubert, M.A., Ritar, A.J., The effect of temperature on the embryonic and larval development of blacklip (*Haliotis rubra*) and greenlip (*H. laevigata*) abalone. Invertebrate Reproduction and Development (in press)

Summary

Embryonic and larval development of *Haliotis rubra* and *H. laevigata* was observed under 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 blacklips and greenlips, respectively. Such information facilitates the prediction of the dispersal window for each species at a given temperature.

Keywords: Biological Zero Point; *Haliotis laevigata*; *Haliotis rubra*; Larval development; Polar bodies; Temperature

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, in prep). 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 blacklips and 12-22ºC for greenlips; 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; Seki and Kan-no, 1977; Kabir, 2001; Sawatpeera et al., 2001). The larval BZP 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.

Methods

Spawning induction
Wild-caught blacklip and greenlip abalone broodstock were induced to spawn using heated, UV-irradiated seawater (Grubert and Ritar, in press). 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).

Experiment 1: Early development and Biological Zero Point (BZP) estimation
Inseminated eggs from both species were held in 70 ml polystyrene jars maintained at ca. 12ºC, 14ºC, 16ºC, 18ºC and 20ºC using 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 10^5 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 line was then extrapolated (using inverse prediction, Zar, 1996) and these values averaged to yield the BZP.

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.

Results

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 1). In blacklips, 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 greenlips 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 blacklips and greenlips in Figs. 1a and b, respectively. The interpolation of these relationships to the x-axis gave larval BZP estimates averaging 7.8 ± 0.0ºC and 7.2 ± 0.1ºC for blacklips and greenlips, respectively (Table 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 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 blacklips and greenlips, respectively.

Discussion

As is the case for several other 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 blacklips and greenlips, respectively, Grubert and Ritar, in prep). 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 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.

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 blacklips and greenlips, respectively. Again, these intervals are similar to that for *H. discus hannai* (390 EATºC-h), but greater than those for *H. 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 blacklips and greenlips, 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, larvae of other species such as *H. rufescens*, are compromised by such delays in metamorphosis (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 asserts that larvae remain benthic during the veliger stage and have a limited capacity for dispersal (see Prince et al., 1987). Among other things, these authors 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. Given this, and the ubiquitous nature of gravity, we are confident that blacklip and greenlip larvae are pelagic and do disperse.

While temperature affects the duration of the pelagic stage, the distance traveled during this period is a function of water movement. Hence, if larvae hatch 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. Some abalone spawn during typhoons (e.g. *H. discus hannai*) resulting in larvae being transported several kilometres (Sasaki and Shepherd, 1995), whereas others (e.g. *H. kamtschatkana*) spawn under still conditions (Breen and Adkins, 1980), greatly reducing the potential for dispersal. Given these apparent differences in spawning cues, models of larval transport for abalone must take into account the conditions under which each particular species spawn 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 released into the wild) needs to be conducted to determine the upper limit of the dispersal window and extent of larval dispersal *in-situ*.

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Table 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 2. Upper and lower 95% confidence intervals (CI) for BZP estimates (in ºC) of selected embryonic and larval stages of blacklip and greenlip abalone.

	Blacklip			Greenlip		
Stage	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		

Table 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

Table 4. Larval biological zero point (BZP) estimates and effective accumulative temperature (EAT) for hatchout and metamorphic competence (MC) of selected Haliotidae.

calculated by Seki and Kan-no (1977) using data from Leighton (1974).
calculated using data from Leighton (1974).
as *Suculus diversicolor aquatilis*, see Geiger (1998) for taxonomic review.
– indicates data not available

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	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

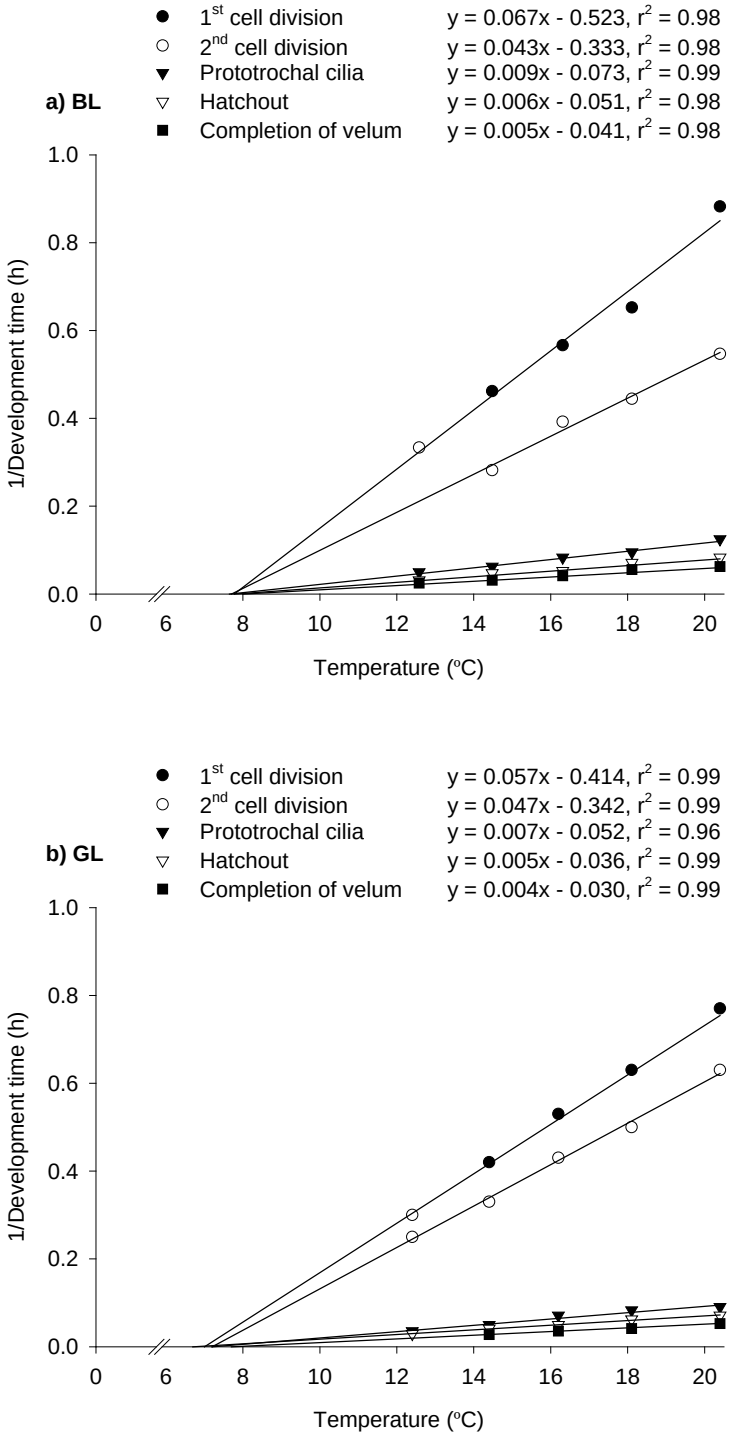

Fig. 1. The relationship between the reciprocal of development time and temperature for selected embryonic and larval stages of (a) blacklip (BL) and (b) greenlip (GL) abalone.

APPENDIX 5 - THE EFFECT OF TEMPERATURE AND SPAWNING STATUS ON THE LIPID AND FATTY ACID COMPOSITION OF CAPTIVE WILD-CAUGHT BLACKLIP (*HALIOTIS RUBRA*) AND GREENLIP (*H. LAEVIGATA*) ABALONE FED A FORMULATED FEED

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The effect of temperature and spawning status on the lipid and fatty acid composition of captive wild-caught blacklip (*Haliotis rubra*) and greenlip (*H. laevigata*) abalone fed a formulated feed. Aquaculture (in press)

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. Half the gravid animals were induced to spawn (using UV-irradiated seawater) and the remainder left untreated. All animals were then sacrificed and samples of foot, digestive gland (DG) and gonad analyzed 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 proportion of LA and EPA in the DG were intermediate between those of the testis and ovary. Differences in the proportion of ARA between the foot, DG and gonad were possibly due to the conversion of ARA to prostaglandins in the latter tissues. 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.

Keywords: *Haliotis laevigata*; *Haliotis rubra*; Lipids; Fatty acids; Temperature; Formulated feed

Introduction

Physical changes in gonad (particularly ovarian) microstructure during gametogenesis of blacklip (*Haliotis rubra*) and greenlip (*H. laevigata*) abalone are well documented (Harrison and Grant, 1971; Shepherd and Laws, 1974; Lleonart, 1992). 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 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 they 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 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 gravid and spent blacklip and greenlip abalone fed a formulated feed. A secondary aim was to determine the effect of two different temperatures (14ºC and 18ºC) on the FA composition of these tissues. 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.

Methods

Collection and inspection of animals
Blacklip and greenlip abalone broodstock 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 induced to spawn using UV-irradiated seawater (Grubert and Ritar, in press). Only animals that spawned or showed no sign of gonad development when first examined were used in the experiment.

Experimental design
Species and sexes were separated and randomly assigned to one of two temperatures (i.e. 14ºC or 18ºC). Blacklips were conditioned for 1450 EATºC-days (equal to 229 and 145 days at 14ºC and 18ºC, respectively) and greenlips for 1800 EATºC-days (equal to 255 and 165 days at 14ºC and 18ºC, respectively). These effective accumulative temperature (EAT) values were chosen as they represent the optimal conditioning interval for each species (Grubert and Ritar, in press). The EAT is the cumulative difference between the daily water

temperature and the biological zero point (the temperature below which gonad development is arrested), which is 7.8ºC in blacklips and 6.9ºC in greenlips (Grubert, unpublished). When each interval elapsed, two animals of each sex were sampled for lipid and FA analysis. At the same time, one male and one female were induced to spawn using UV-irradiated seawater and the spent animals sampled within two days of spawning. For each sex, spent animals from both temperature treatments were combined in the analysis.

Husbandry and monitoring
Species and sexes were conditioned in separate 150 L fiberglass tanks receiving flow-through seawater at a 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). 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).

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 refrigerator 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.

Quantification of lipids and fatty acids
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 (in 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 a 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 analyzed 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 /sec and mass range 40–650 Dalton.

Results

Analysis of formulated feed
Total lipid and moisture content of the formulated feed was 4.8 ± 1.6% (of 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 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.

Analysis of abalone tissues
There was no obvious effect of temperature or spawning status on the lipid or FA composition of blacklip (BL) or greenlip (GL) tissues. 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 (Table 2). In general, the moisture content of male tissues was less than that of female tissues, except in the gonad where the reverse was true. 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%, pooled across sex and species) and ovary (18–26%, pooled across species). The variation in LA was largely responsible for the difference in n-3:n-6 ratios between tissues (Tables 3–6). 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 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 decrease in the ARA:EPA ratio between these tissues. The magnitude of this difference was greater in males, as the testis contained approximately ten times more EPA than the ovary. In BL, 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 GL.

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 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 content (and FA composition) between spent and gravid animals. More intensive sampling of spent

animals (i.e. over several weeks post-spawning) needs to be conducted to validate this assertion.

The formulated feed provided to *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 variation 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 larger fraction of palmitic acid was offset by lower proportions of 18:1n-9, 18:1n-7 and 22:5n-3 in the samples analyzed by Su et al. (2004). The reasons behind these differences are unclear.

When foot muscle FA profiles of *H. rubra* and *H. laevigata* fed a formulated feed are compared to that of *H. fulgens* fed macroalgae (Nelson et al., 2002a), several differences emerge. One is the lower proportion of SFA (primarily 16:0) in the foot of *H. rubra* and *H. laevigata* (about 28%) compared to that of *H. fulgens* (39%). Another being the smaller fraction of ARA in the former (about 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 analyzed 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. With the exception of ARA, these figures are different to those of the formulated feed as well as the other tissues, suggesting that some FA are synthesized or converted in the 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 may to be able to retroconvert DHA to form EPA (Dunstan et al., 1996). This explains 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 this 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).

As with female *H. fulgens* (Nelson et al., 2002a), we observed a decline in the proportion of ARA between the foot, DG and gonad of female *H. rubra* and *H. laevigata.* The same pattern was also observed in males of both species. These findings support Nelson's proposition that ARA is converted to prostaglandins (which are important in spawning of abalone; Morse et al., 1977) in the DG and ovary, and infer that the same process takes place in the testis.

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 (in press) were fed the same commercially available broodstock feed. However, the proportion of LA in freshly-spawned ova (1–7%, Daume and Ryan, in press) was lower than in ovarian tissue (19%; this study). Possible reasons for this difference include the retention of LA in ovarian supportive tissue and regional variations in FA metabolism, as broodstock for each study were taken from sites approximately 3000 km apart.

While culture temperatures of 14ºC and 18ºC have significant effects on the rate of gonad maturation in blacklip and greenlip abalone (Grubert, unpublished), the lack of any effect on the FA profiles suggests 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 and 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 (14ºC and 18ºC) was insufficient to elicit any change in the lipid or FA composition. Hence, a wider range of experimental temperatures may be needed to induce compensatory changes in 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. The low levels of ARA in the DG and gonad suggest that ARA is converted to prostaglandins in these tissues. Given this, it is possible that supplementation of abalone broodstock feeds with ARA may improve spawning performance. Further work, including more intensive post-spawning sampling, needs to be conducted to fully understand the lipid and FA dynamics during gametogenesis.

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Table 1. Percentage (mean ± S.E; n = 5) fatty acid composition of the artificial 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 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.

		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 3. Percentage fatty acid composition (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. Percentage fatty acid composition (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 5. Percentage fatty acid composition (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 6. Percentage fatty acid composition (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).

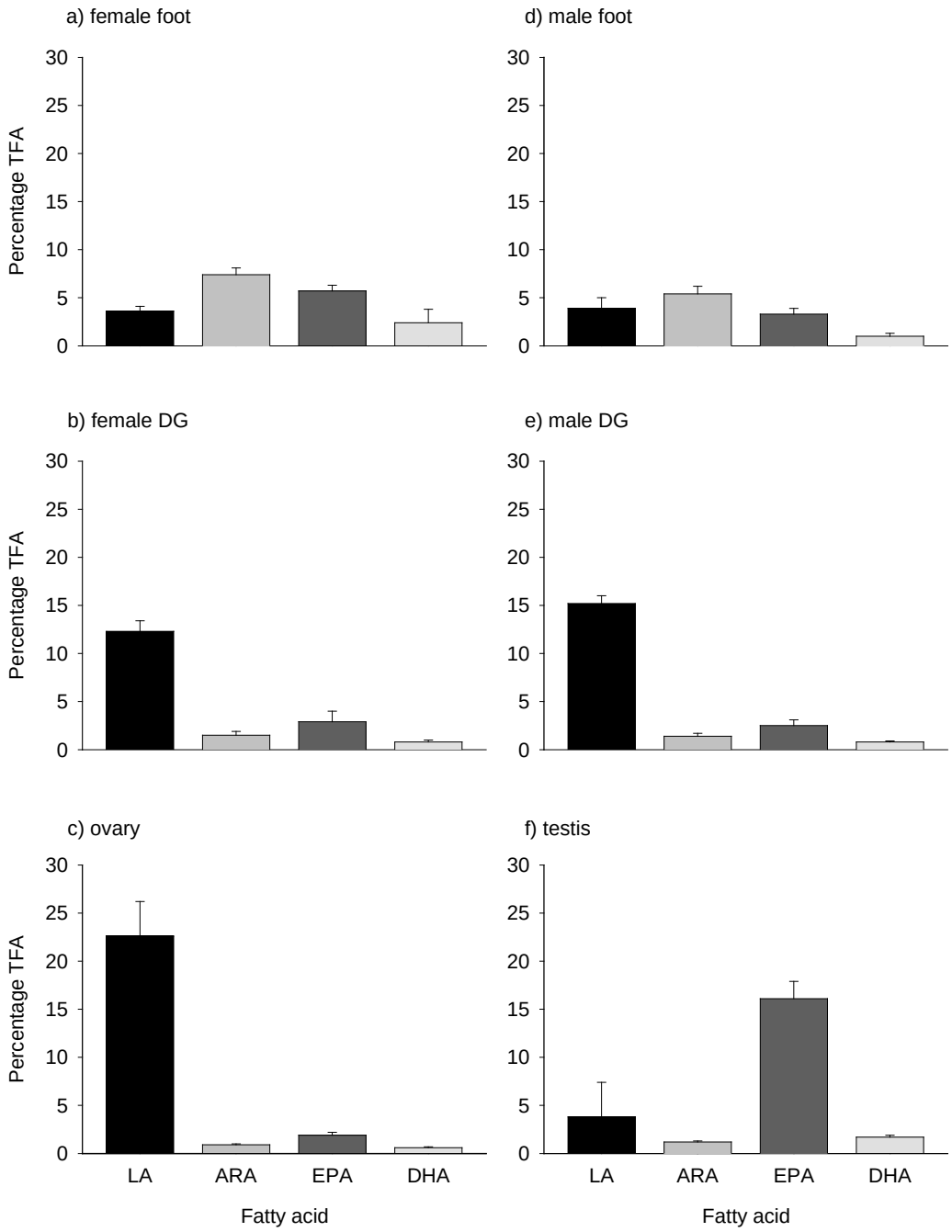

Fig. 1. Percentage total fatty acid (TFA; mean ± S.E.) of linoleic (LA, 18:2n-6), arachidonic (ARA, 20:4n-6), eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:6n-3) acids in the foot, digestive gland (DG) and gonad of female (a–c) and male (d–f) blacklip abalone. Data pooled for temperature and spawning state.

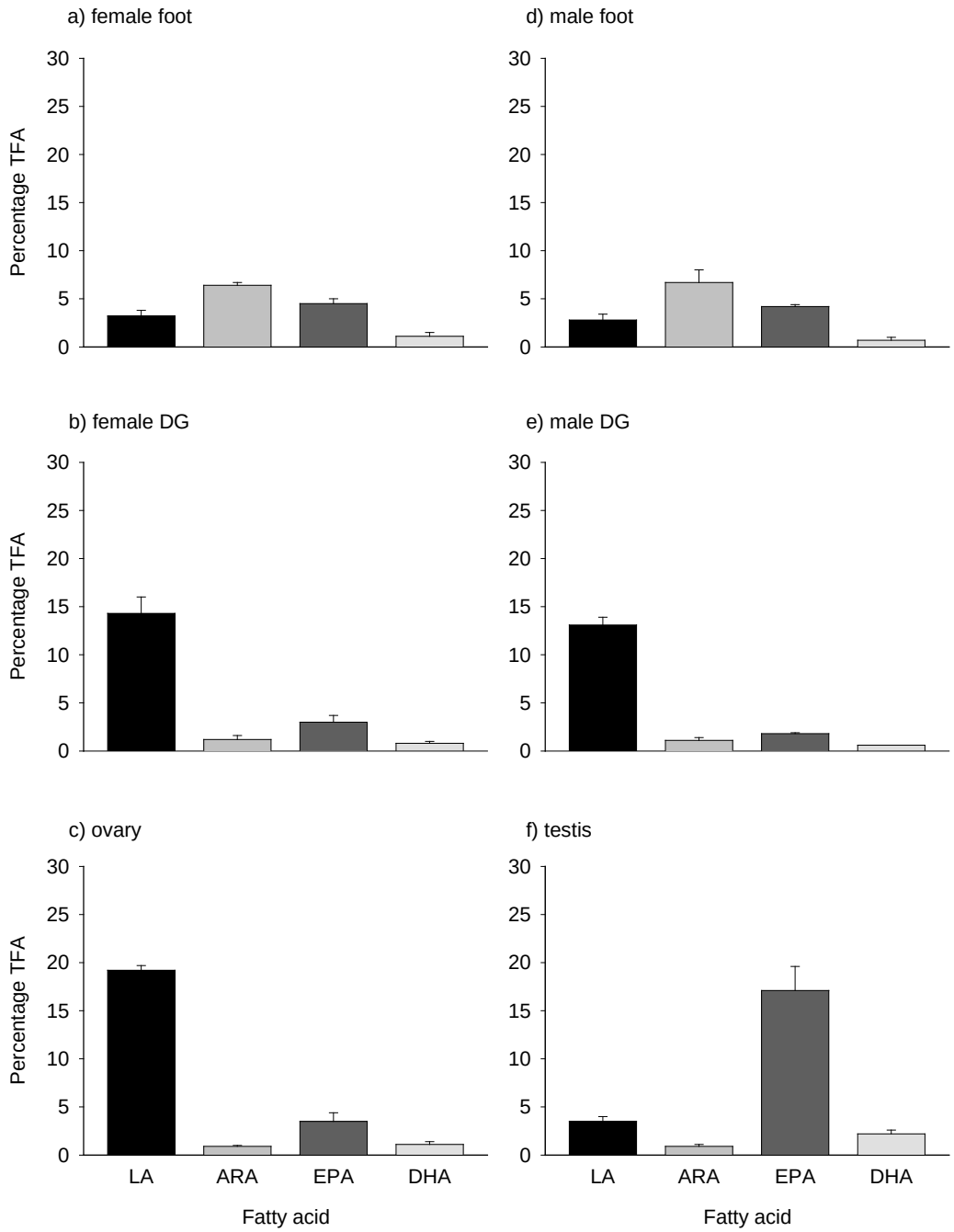

Fig. 2. Percentage total fatty acid (TFA; mean ± S.E.) of linoleic (LA, 18:2n-6), arachidonic (ARA, 20:4n-6), eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:6n-3) acids in the foot, digestive gland (DG) and gonad of female (a–c) and male (d–f) greenlip abalone. Data pooled for temperature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

APPENDIX 6 - FERTILISATION OF EGGS FROM BLACKLIP (*HALIOTIS RUBRA*) AND GREENLIP (*H. LAEVIGATA*) ABALONE IS DETERMINED BY THE DENSITY AND DURATION OF CONTACT WITH SPERMATOZOA.

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This chapter submitted as:
Fertilisation of eggs from blacklip (*Haliotis rubra*) and greenlip (*H. laevigata*) abalone is determined by the density and duration of contact with spermatozoa. Journal of Experimental Marine Biology and Ecology

Abstract

A factorial experiment, in which blacklip (*Haliotis rubra*) and greenlip eggs (*H. laevigata*) 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 that both factors had a significant effect on fertilisation success. There was also a significant interaction between these factors in both cases. 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. 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. Light absorbance at 340 nm was highly correlated to sperm density in both species, providing a simple, rapid and accurate alternative to haemocytometer counts.

Keywords: *Haliotis laevigata*; *Haliotis rubra*; sperm density; light absorbance; sperm-egg contact time; sperm morphology

Introduction

Blacklip (*H. rubra*) and greenlip (*H. laevigata*) 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 produce up to 10^{12} sperm (Babcock and Keesing, 1999; Grubert and Ritar, submitted).

Fertilisation success of free-spawning marine invertebrates, such as abalone, is reliant not only on prolific gamete production, but also adult density. 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). This arises from the rapid dilution of the sperm plume and the fact that abalone eggs are negatively buoyant, which restricts their time in the water column. In extreme cases, animal density may be so low that sperm have little chance of fertilizing the eggs, a phenomenon known as the Allee effect (Allee, 1931).

Sperm age affects fertilisation success in abalone (Babcock and Keesing, 1999; Baker and Tyler, 2001). Hence, artificial insemination of eggs must proceed rapidly so as to reduce confounding by this factor. However, determining sperm density (for example in a hatchery) can be slow, particularly if sperm from several males are to be used and/or labour is limited. Sperm density is typically assessed using a haemocytometer, but Kikuchi and Uki (1974) showed that the process can be automated using a spectrophotometer. The ability to rapidly estimate sperm density from light absorbance thus reduces the effect of sperm age during studies of haliotid fertilisation kinetics.

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 occurs when the concentration of sperm lysin becomes so high that the vitelline membrane is completely dissolved, leading to low or no fertilisation. 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 establish the relationship between sperm density and light absorbance; secondly, to examine the combined effects of sperm density and contact time on the fertilisation rate; and finally, to describe sperm morphology (using scanning electron microscopy). These factors are discussed in relation to potential differences in fertilisation rate between species.

Methods

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.

Relationship between sperm density and light absorbance
For each of three different males of each species, sperm water 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. A series of absorbance readings, haemocytometer counts and two-fold dilutions were repeated until absorbance fell to the lowest detectable level (0.001).

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, all crosses in duplicate) in which eggs were exposed to different sperm densities then removed and rinsed at predetermined time intervals. PVC tubes 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. A positive control, in which eggs were exposed to an aliquot of stock solution (i.e. 1×10^7 and 0.4×10^7 sperm ml^{-1} for blacklips and greenlips, respectively) 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. 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 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.

Preparation and examination of sperm and embryos using scanning electron microscopy
Sperm of each species were centrifuged at 8000 x for 15 min and the pellet transferred to 5% glutaraldehyde sucrose-phosphate buffer (pH 7.4) solution for 18 h at 4ºC. Each sample was then placed in a 0.5 ml porous vessel 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.

Statistics
Percentage data were arcsine transformed prior to analysis. Two-way Analysis of Variance (ANOVA) with replication was used to test for significant differences in fertilisation rate between treatments (i.e. sperm-egg contact time and sperm density). One-way ANOVA and Tukey-Kramer HSD tests (for post-hoc comparison) were used locate significant differences between contact times. Bartlett's tests were used to check for homogeneity of variance. Probabilities of <0.05 were considered significantly different.

Results

Relationship between sperm density and light absorbance
In both species, the relationships between sperm density and light absorbance (at 340 nm) were power functions with high correlation coefficients (Fig. 1). These functions were used to estimate sperm density from absorbance in subsequent fertilisation trials.

The effect of sperm-egg contact time and sperm density on fertilisation of blacklip abalone (H. rubra)
Both sperm-egg contact time and sperm density had a significant effect on fertilisation success of blacklip eggs. There was also a significant interaction between these factors (Two-way ANOVA; $p < 0.001$ for all factors). 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. 2a). 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 1×10^5 sperm ml^{-1}, fertilisation rate increased proportionally with contact time, ranging from 36–96% (Fig. 2b). At 1×10^6 sperm ml^{-1}, fertilisation success was 51% after 7 s contact and increased to (and remained at) ≥ 95% by 480 s and thereafter (Fig. 2c). At the highest sperm density of 1×10^7 sperm ml^{-1}, fertilisation was 72% after 7 s contact and rose to ≥ 90% by 60 s, where it remained for all other treatments except the 1200 and 4800 s groups in which fertilisation was nil (the contact time x density interaction). 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.

The effect of sperm-egg contact time and sperm density on fertilisation of greenlip abalone (H. laevigata)
Similar to blacklips, both sperm-egg contact time and sperm density had a significant effect on fertilisation success of greenlip eggs. A significant interaction was also detected in the greenlip data (Two-way ANOVA; $p < 0.001$ for all factors). Short sperm-egg contact times (i.e. 7–60 s) at the lowest sperm density (0.4×10^4 sperm ml^{-1}) resulted in low (< 10%) fertilisation success (Fig. 3a). When contact time was increased beyond one minute, fertilisation rate rose proportionally, reaching 63% at 2400 s. An increase in sperm density to 0.4×10^5 sperm ml^{-1}, saw the fertilisation rate rise by 20–30% across all contact times and reach > 95% by 1200 s (Fig. 3b). At the two highest sperm densities (i.e. 0.4×10^6 and 0.4×10^7 sperm ml^{-1}), fertilisation rates were > 75% for all treatments (Figs. 3c and 3d), the exceptions being the three longest exposure times of 480, 1200 and 2400 s at 0.4×10^7 sperm ml^{-1}, where fertilisation was nil (the contact-time x density interaction). 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.

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. 4. The total length of blacklip abalone sperm 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 1). Corresponding lengths for greenlip abalone sperm 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, it shape was slightly different, blacklips having a blunt tip and greenlips a V-shaped tip.

Discussion

This study showed that the fertilisation rate of blacklip and greenlip eggs increased proportionally with gamete contact time at sperm densities between 10^4 and 10^7 ml^{-1} (with the exception of the contact-time x density interaction). The extent of this increase was greatest in blacklip eggs, particularly at sperm densities of 10^4–10^6 ml^{-1}. By contrast, fertilisation rate of greenlip eggs exposed to sperm at 0.4×10^6 ml^{-1} showed little variation, being ≥ 75% regardless of contact time. Eggs of both species were destroyed by polyspermy when exposed to concentrated sperm (ca. 10^7 ml^{-1}) for long periods (i.e. ≥ 1200 s for blacklips and ≥ 480 s for greenlips).

The lowest sperm concentration that ensured high fertilisation at short contact times (i.e. the optimal sperm density - OSD) was 1×10^6 ml^{-1} for blacklip eggs and 0.4×10^6 ml^{-1} for greenlip eggs. Our OSD estimate for *H. rubra* contrasts markedly with that of Huchette et al. (2004) who observed polyspermy at 1×10^6 sperm ml^{-1} (30 s contact time) and reported an OSD of 5×10^3 to 1×10^5 sperm ml^{-1}. Possible reasons for this difference include variable sperm efficacy and/or egg fertilisation potential.

A previous work on the fertilisation biology of *H. laevigata* (Babcock and Keesing, 1999) showed that sperm density, but not sperm-egg contact time, had a significant effect on fertilisation success of this species but our data suggests that both factors are important. We attribute this difference to the fact that we replaced used sperm solutions with freshly mixed suspensions following each contact time trial, whereas Babcock and Keesing (1999) did not. Our method avoided the potential confounding effects of sperm age which, in the case of Babcock and Keesing (1999), may have masked the effect of sperm-egg contact time on fertilization rate.

Much emphasis has been placed on the importance of sperm density and sperm-egg contact time on fertilisation of haliotids, while 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 (5.3×10^7 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. This being the case, 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 storms or typhoons (e.g. *H. discuss hannai*; Sasaki and Shepherd, 1995) the effect of the attractant would be negligible. In 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.

Egg size is another factor which may influence fertilisation success of abalone. Indeed, both Litaay and De Silva (2001) and Huchette et al. (2004) describe a relationship between yolk (≈ cytoplasm) diameter and fertilisation success for blacklips. However, the former authors documented a quadratic function whereas the latter reported an exponential function (derived from the relationship between sperm density for 50% fertilisation and cytoplasm diameter; Huchette et al., 2004). Conflicting results, and the fact that both works used small ($n < 20$) data sets and either a narrow size range (Huchette et al., 2004) or an unbalanced data set (Litaay and De Silva, 2001) means that the results of these studies should be applied with caution. Clearly, more work is required to resolve the role of egg size on fertilisation success of abalone.

Both total and component length of abalone sperm differ between species, with blacklip and greenlip abalone sperm being of intermediate size (see Table 1). While species with a larger acrosome (containing more sperm lysin) and/or a longer flagellum (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).

The optimal sperm density for greenlip abalone was less than half that for blacklip abalone. This may be because the acrosome of greenlip sperm is V-shaped, and perhaps more effective at penetrating the jelly coat, than the blunt tip of blacklip sperm. Differential efficiency of the acrosome reaction and/or vitelline membrane permeability may also explain variations in fertilisation success between species.

It is also interesting to note that sperm production of greenlip abalone is 10- to 100-fold less than that of equivalent sized blacklip abalone (Grubert and Ritar, submitted). Thus, high fertilization in greenlips at a 10-fold lower sperm density may have evolved as a mechanism to overcome low sperm production, or vice versa, high fertilization rates at low sperm density may have evolved to allow less investment in sperm production.

There was a close correlation between light absorbance and sperm density in both blacklip and greenlip abalone. Kikuchi and Uki (1974) previously documented a curve relating light absorbance to density of *H. discus hannai* sperm, and while they did not provide an equation, it appeared to be a power function. Spectrophotometric estimation provides an accurate, simple and rapid means of sperm quantification, readily applied in either the laboratory (as demonstrated here) or the hatchery.

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 (< 30 s) contact times have no detrimental effect on fertilization success of either species, but prolonged exposure (i.e. ≥ 20 min) to concentrated sperm results in polyspermy. This study demonstrates that blacklip and greenlip sperm concentration correlates with light absorbance (at 340 nm) and that sperm density of both species can be rapidly and accurately assessed using a spectrophotometer. Differences between species in acrosome shape and the OSD for fertilisation suggest that sperm morphology may influence fertilisation success. 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.

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Table 1. Comparison of dimensions of sperm components in selected Haliotidae. Dash indicates data not available.

	Sperm component length x diameter (µm)					
Species	Acrosome	Nucleus	Midpiece	Flagellum	Total Length	Source
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.

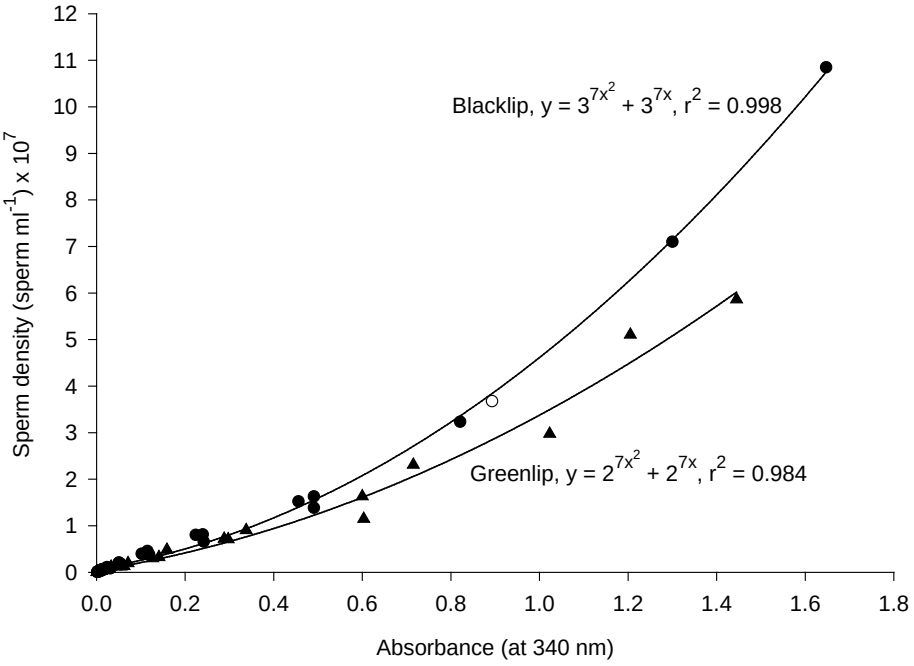

Fig. 1. Relationship between light absorbance at 340nm (x) and density (y) for blacklip and greenlip abalone sperm.

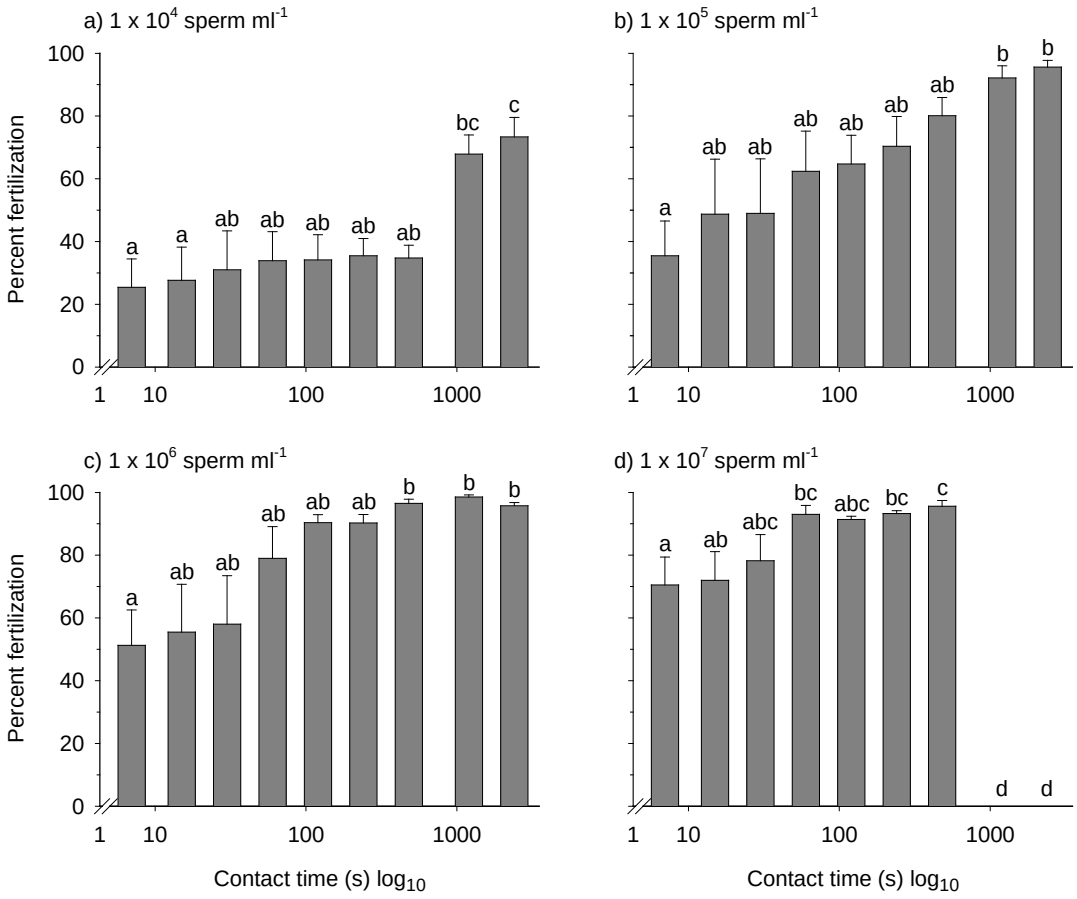

Fig. 2. The effect of sperm-egg contact time at sperm densities of a) 1 x 10^4 sperm ml^{-1}, b) 1 x 10^5 sperm ml^{-1}, c) 1 x 10^6 sperm ml^{-1} and d) 1 x 10^7 sperm ml^{-1} on fertilisation success of *H. rubra*. Bars with the same letters within each density treatment are not significantly different.

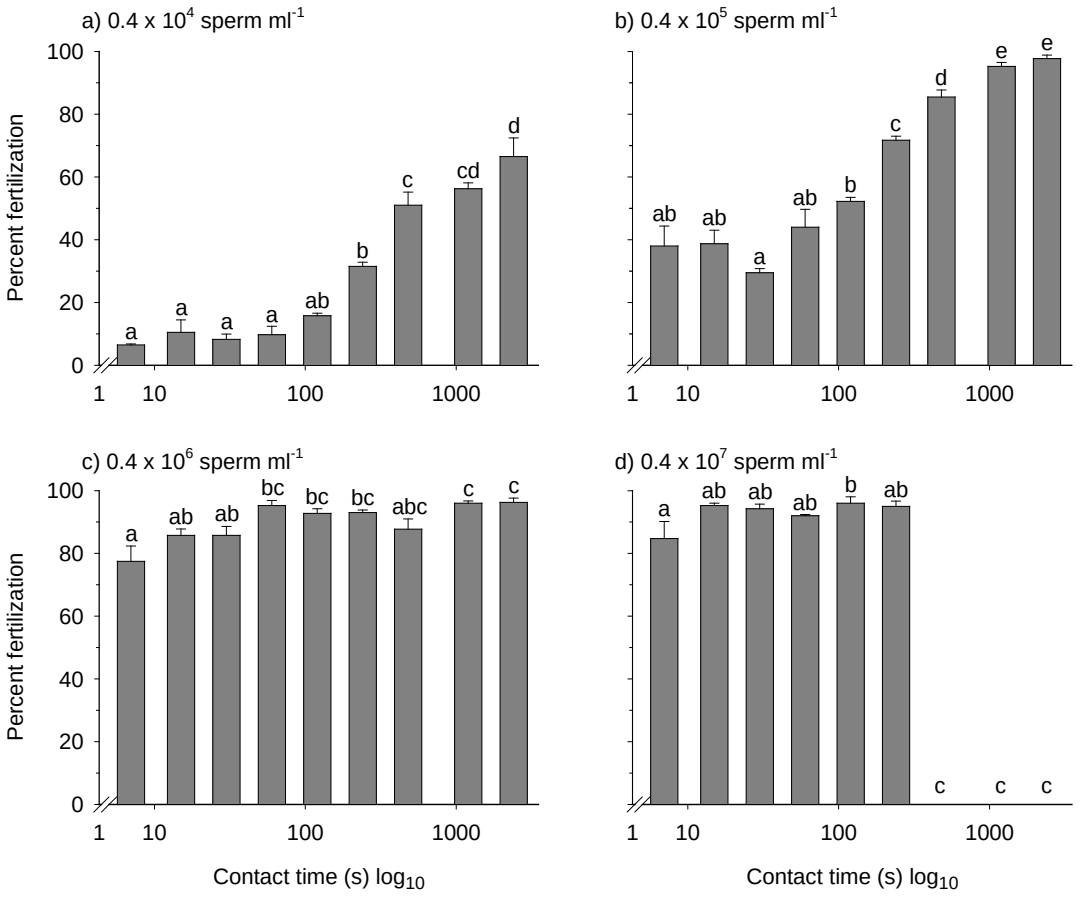

Fig. 3. The effect of sperm-egg contact time at sperm densities of a) 0.4 x 10^4 sperm ml^{-1}, b) 0.4 x 10^5 sperm ml^{-1}, c) 0.4 x 10^6 sperm ml^{-1} and d) 0.4 x 10^7 sperm ml^{-1} on fertilisation success of *H. laevigata*. Bars with the same letters within each density treatment are not significantly different.

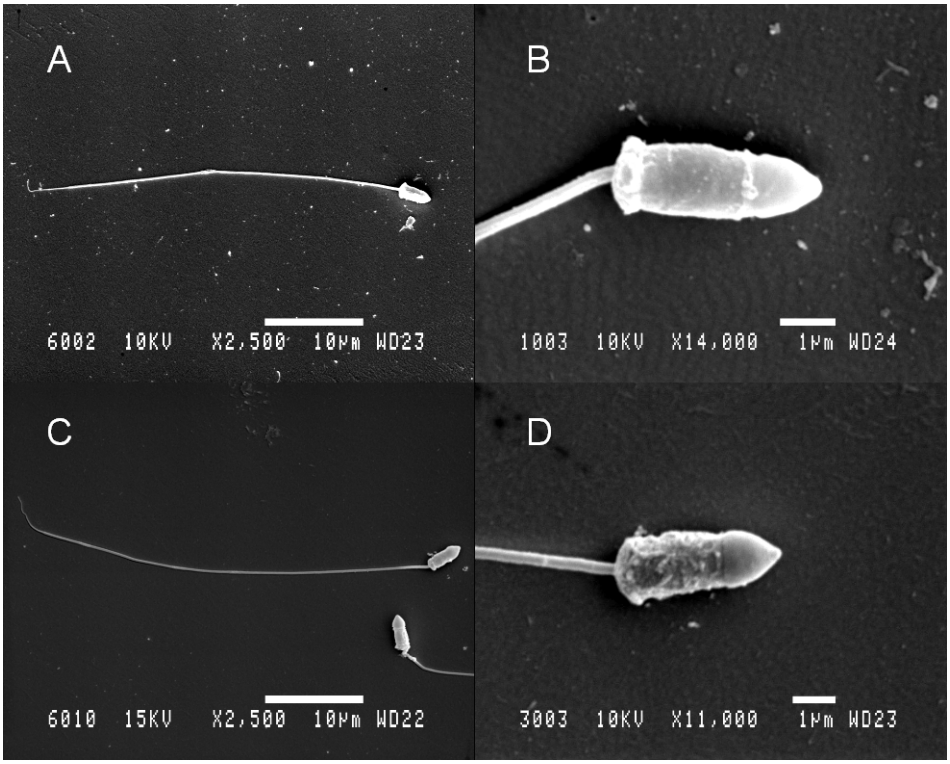

Fig. 4. Scanning electron micrographs of (A–B) blacklip and (C–D) greenlip sperm.