Spawning and larval rearing research on King George whiting *(Sillaginodes punctata)* relevant to aquaculture and fisheries biology

JM Ham and WG Hutchinson

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SOUTH AUSTRALIAN RESEARCH AND DEVELOPMENT INSTITUTE

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Objectives

1. Establish two breeding stocks of King George whiting (KGW) and control egg production from one stock during the natural spawning season and from the other stock six months out-of-season.
2. Identify spawning frequency and periodicity to provide an accurate estimate of fecundity for fisheries models.
3. Complete intensive larval rearing investigations to define environmental tolerances of larvae and to expose and solve problems during the larval culture period.
4. Document the behaviour of larvae in the water column at different stages of development.
5. Assess the potential of larval culture of KGW for commercial aquaculture.

Non - technical summary

Outcomes achieved to date

The principle outcome of this project was to present results of the first systematic investigation on the reproduction and development of KGW eggs and larvae. This involved investigating the effect of environmental parameters on the growth and survival of KGW during their early development and documenting fundamental biological information that is beneficial for the culture of this species and for fisheries research.

This project also identified and documented the occurrence of the disease *Exophiala salmonis*, not previously known to occur in this species.

The degeneration of post-ovulatory follicles has been reported, which will assist fisheries scientists to identify the time of spawning, spawning frequency and consequently estimates of spawning biomass.

Completion of this project has clearly identified the challenges that need to be addressed if commercial aquaculture of KGW is to progress.

Chapter 1

KGW is a popular marine finfish highly prized in southern Australia by fishers and consumers. Interest in the potential for aquaculture of KGW for both commercial markets and re-stocking programs prompted an assessment of KGW. Evaluating the aquaculture potential of KGW provided an opportunity to investigate the frequency and periodicity of KGW spawning which has been identified in previous FRDC-funded research as required to accurately estimate the fecundity of this species.

To establish breeding stocks and investigate controlled egg production of this species, KGW were captured off the coast of Kangaroo Island and transferred to SARDI, where two separate breeding stocks (one housed in outdoor flow through tanks and the other in a controlled environment broodstock facility) were established. Natural spawning (release of eggs voluntarily without induction) and hormone-induced spawning (the use of commercial hormones to promote spawning) were evaluated in both stocks of fish. Spawning dysfunction was experienced in both natural and out of season stocks, where final oocyte maturation (FOM) was unpredictable or absent. Spontaneous spawning was unreliable, egg production was low and the fertilisation rate was erratic varying between 0% to 90% but was typically less than 50%. This type of spawning dysfunction is common in captive finfish broodstock. This suggests that, for this species, greater control over egg production (i.e. timing, quantity and quality) is required for a commercial hatchery operation.

A fungal pathogen, *Exophiala salmonis* not previously seen in KGW was identified in breeding stocks during this study. Clinical outbreaks of *Exophiala salmonis* have been seen regularly in farmed salmonids in the northern hemisphere. The outbreak of this pathogen appears to have compromised some spawning trials.

Chapter 2

To identify spawning frequency and periodicity of KGW, two methods were trialed.

The first method involved visual assessment of captive female broodstock during the spawning season. Broodstock were attributed a score (stage 1 to 4) based on the degree of swelling in the abdominal area, which is used as an indication of the reproductive stage of the fish. This method assumes that a progression from stage 4 (very prominent swelling to the extent where muscle striations can be seen on the sides of the abdomen in the vicinity of the gonadal pore) to stage 1 (no visible development in the dorsal side (belly) of the fish) is the result of a spawning event. This method also assumes that spawning frequency in captivity is a true representation of spawning frequency in the wild. This was found to be an unsuitable method of estimating spawning frequency and periodicity, primarily due to the spawning dysfunction that is experienced in captive KGW broodstock.

The second method involved taking histological sections of ovaries at known times after strip spawning (3-36 hours) to document the deterioration of post-ovulatory follicles (POF) within an ovary. Documenting the deterioration of POF allows the timing of spawning in KGW to be estimated within 6 hours from POF structures at 18.5°C.

Chapter 3

To investigate the larval rearing of KGW a series of environmental parameters were assessed and developmental stages documented.

The development of oocytes and fertilised eggs were documented from a primary oocyte stage to hatch.

The effect of water temperature and salinity on the hatching and survival of KGW eggs was evaluated. Results indicated that temperature and salinity had a combined effect on the hatching and survival of KGW eggs with maximum hatching success being trials 16°C to 22°C and with salinities between 35 to 45 during incubation.

Larval development was documented from yolk sac stage (hatch) until post-flexion (29-50 days post hatch).

Clearing and staining techniques were used to determine the mouth gape of KGW larvae at first feeding, investigate the relationship between mouth gape and standard length and to investigate the skeletal structure of a captive KGW juvenile. The mean mouth gape at first feeding ranged from 480µm to 670µm. A relationship was established between standard length and mouth gape (R= 0.8129) and this can be used as a guide to the feed size able to be ingested by larvae at first feed. Results indicate that as larvae grow and they become more efficient predators they are able to ingest prey items that represent a greater proportion of their mouth gape and hence deviate from the relationship.

The skeletal structure of KGW juveniles reared in captivity is consistent with those documented for wild KGW.

The effect of salinity on the hatching success, yolk sac absorption, larval size, mouth gape at first feeding and the period of survival during starvation were investigated in larval KGW. No significant difference was detected in any of the parameters investigated, but some concerns exist in the results due to sub-optimal larval quality in some experiments.

The timing at which KGW larvae are weaned from rotifers to *Artemia* was investigated to identify the stage at which KGW larvae are able to ingest *Artemia* with respect to mouth gape and maximise growth and survival. Results suggest that the functional ability of larvae to ingest prey may not correlate with their ability to digest *Artemia* efficiently. Improved growth and survival of larvae was obtained when larvae had attained a standard length of 10.0mm.

The growth and survival of KGW fingerlings (120dph) at increased water temperatures (18°C-26°C) was investigated over a 70-day period. Results showed that temperature affected the growth of KGW over the period of the trial. Maximum growth rates were obtained between 22°C and 26°C.

Further trials investigated the effect of temperature on the growth of KGW juveniles (Trial 1-205dph and trial 2-146dph). The growth, survival and food conversion rate (FCR) of KGW juveniles were investigated at increased water temperatures (20°C -26°C) to evaluate the optimum temperature and possible growth rate of KGW juveniles through to market size. The FCR recorded at all water temperatures was excessive (2.91-5.10) indicating that KGW are a difficult species to feed efficiently. No significant difference was determined between temperature treatments (20°C -26°C). A crude estimate of time to market size from 1g based on a projection of average growth rates during trials reveals marginal difference between temperature treatments 20°C, 23°C and 26°C (estimated time taken being 13.2 months, 12.5 months and 12.0 months respectively).

Chapter 4

The behaviour of larvae in the water column during the larval culture period was not completed specifically as larvae used in larval rearing trials did not respond to transfer between tanks.

Observations from larval rearing trials have shown that KGW inhabit the surface and sides of tanks until metamorphosis that generally occurred from 50-70 dph (14-20mm standard length) at water temperatures 20°C -24°C. Coincident with this change in morphology there was a distinct change to a mostly bottom-dwelling behaviour.

Chapter 5

The potential of KGW for commercial aquaculture was investigated based on the trial results and market potential of KGW.

Throughout this research project it has been demonstrated that KGW beyond the juvenile stage are durable under culture conditions and are not cannibalistic at any stage of development. In addition, KGW are easily handled and have shown minimal incidence of disease infections or parasite infections. These features should provide benefits for husbandry operations required during hatchery and on-growing to market size.

Although this species has some features that provide advantages for aquaculture several challengers were encountered in this study that affect the commercialisation potential of KGW. The main problems encountered were:

1. inability to achieve a controlled supply of high quality eggs from broodstock,
2. poor larval survival in the hatchery and
3. the slow growth of KGW throughout both the larval and grow-out periods.

The principle output of this project was the production of the Coastal Finfish Hatchery Manual: King George whiting *(Sillaginodes punctata)* in CD-Rom and hard copy formats. The hatchery manual presents protocols developed and incorporates preliminary results of research trials conducted to date. This manual aims at covering the fundamental aspects of hatchery production for marine finfish in general using KGW as the focus species. The areas covered in this manual include: broodstock systems, broodstock husbandry, egg biology, controlled egg production, larval rearing including live feed production, nursery and subsequent grow-out, health and diseases.

KEYWORDS: King George whiting; Finfish; Hatchery culture; Larval rearing

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We wish to gratefully acknowledge all of the people who provided assistance at various stages through the project. Technical assistance was provided by Donna Cahill, Shaun McCarthy and Paul Drummond. Craig Noell provided advice on the staining of larvae and juveniles. Steve Clarke provided general scientific advice particularly during the editing phase. Dr Tony Fowler assisted in the design and sampling of the post-ovulatory follicle study. Jeff Buchanan provided advice on broodstock nutrition. Dr Ruth Reuter (IDEXX Veterinary Pathology Services) assisted with identifying and reporting the disease outbreak *Exophiala salmonis*. Advice on statistical procedures was provided by Debra Partington (Biometrics SA). The first draft of the report was improved substantially by comments from the two reviewers Dr Milena Fernandes and Dr Richard Musgrove and by editorial comments from Lyn Ham.

We also wish to thank Spencer Gulf Aquaculture, Posaqua, and Coorong Council for their co-operation and assistance with preliminary commercial grow-out trials.

Finally we wish to acknowledge the South Australian Government and the Fisheries Research and Development Corporation for their financial support.

Background

KGW, *Sillaginodes punctata* has "icon" status in South Australia with a concomitant high market value ($45/kg fillets and up to $19.80/kg whole fish; 2003 retail), which creates interest as a potential species for marine finfish aquaculture.

Until this project, there has been no systematic attempt to investigate the potential of KGW as a commercial aquaculture species.

In May 1996, for the first time SARDI at the SA Aquatic Science Centre (SAASC), induced KGW to spawn, fertilised eggs were hatched and a small number of larvae were reared through to metamorphosed juveniles. These results suggested that commercial success might be achieved with further research and development to provide, identify and address a wide range of issues that became apparent as part of this preliminary study.

Studies of reproductive biology to assist controlled egg production and larval rearing is an essential area of research for the aquaculture development of any 'new' marine finfish

species. For SARDI this presented a good opportunity to conduct collaborative research between aquaculture and fisheries scientists who seek to understand the spawning and larval recruitment of KGW.

This project aimed to define optimal environmental parameters (water temperature; salinity) for egg incubation and larval rearing and develop suitable larval rearing protocols based upon those employed by commercial marine finfish hatcheries.

Need

KGW is the highest valued marine finfish species in South Australia after southern bluefin tuna (*Thunnus maccoyii*). This species attracts a high market price on local and interstate markets, which suggests that it might have good potential as a candidate for aquaculture. To allow commercialisation of this species, there was a need to commence research to control egg production and conduct larval rearing trials, followed by grow-out trials to expose and solve culture problems so that a valid assessment of this species could be provided to aquaculturalists and investors.

Preliminary market research showed that considerable opportunities exist to increase supply of this highly regarded species into the Sydney and Melbourne fish markets. Sydney in particular is a large national market, which is presently poorly serviced with supplies of this species, there is also no opportunity to supply additional products from the wild for this species without considerably increasing pressure on what is generally classified as a fully utilised wild fishery.

Many State Governments of southern Australia also believe there is a need to broaden the base of the aquaculture industry in their regions. KGW has potential as a species more suited to land based culture systems, which will not compete for sites with other species more suited to sea cage culture systems. These developments will allow further expansion of the industry. Aquaculture has a history of poor decisions being taken by misinformed investors. There is a need for this type of development orientated research projects to be undertaken to provide information upon which investors can base decision-making.

Objectives

1. Establish two breeding stocks of KGW and control egg production from one stock during the natural spawning season and from the other stock six months out-of-season (refer to Chapter 1).

2. Identify spawning frequency and periodicity to provide an accurate estimate of fecundity for fishery models (refer to Chapter 2).

3. Complete intensive larval rearing investigations to define environmental tolerances of larvae and to expose and solve problems during the larval culture period (refer to Chapter 3).

4. Document the behaviour of larvae in the water column at different stages of development (refer to Chapter 4).

5. Assess the potential of larval culture of KGW for commercial aquaculture (refer to Chapter 5).

General Methods

At the commencement of this project mature fish (n=534, mean weight=370g) were collected with the assistance of professional fishers from known spawning areas off the north east coast of Kangaroo Island. Fish were held in 7000L flow through seawater tanks onboard the SARDI research vessel 'Ngerin' and transferred to culture facilities at the SA Aquatic Science Centre (SAASC) at West Beach, Adelaide to establish two separate spawning stocks of mature KGW. Additional stocks were captured in September 1999 (n=100; mean weight=300g) and April 2001 (n=177; mean weight=360g) from Pt.Victoria, South Australia and transferred to SAASC, to cover different components of the project.

During each spawning season one tank of broodfish were spawned using modified hormone induction procedures developed for snapper and used successfully for KGW in 1996 (Hutchinson, pers. comm.). These fish were observed each evening and morning to determine which females are likely to, or had spawned. Time of spawning was estimated from the presence of eggs in collectors at the outlet of each tank. A second tank contained broodstock that were maintained without induction under ambient conditions to allow the natural spawning (voluntary – without induction) of KGW to be investigated.

To investigate out-of-season spawning a controlled environment broodstock facility was constructed. 12 months after capture mature KGW were transferred into 4 x 10,000L tanks supplied with recirculating seawater. The water temperature and photoperiod was adjusted to condense variations in an attempt to promote spawning 4 months in advance of the natural season.

Fertilised eggs were used by aquaculture research staff to conduct larval rearing investigations for KGW. An experimental rearing system of 20 x 33L hemispherical fibreglass (FRP) tanks was constructed and used to determine environmental tolerances (i.e. water temperature, salinity, dissolved oxygen) of KGW larvae. Conventional larval rearing was undertaken on a small scale to document significant stages of development and assess growth and survival of larvae at critical phases of commercial hatchery production.

Fingerlings produced during the project were used in preliminary grow-out trials. These trials investigated the effect of temperature (20 - 26°C) on the growth and survival of KGW juveniles grown in research and commercial scale systems. This growth data provide preliminary information on the time taken for KGW juveniles to reach market size. This is fundamental information contributing to an assessment of the economic viability of KGW as a commercial aquaculture species.

Statistics were performed in consultation with the Biometrics Department, SA.

Chapter 1 Broodstock: controlled egg production

1.1 Natural and induced spawning of KGW broodstock in captivity

Introduction

For both research and commercial hatchery operations a controlled supply of high quality eggs is required. This involves the production of large amounts of eggs at one time. To achieve this in small fish with relatively low batch fecundity, such as KGW, (fecundity estimates ranging between 5,250 to 152, 191 hydrated oocytes per ovary; Fowler *et al*, 1999) large numbers of broodstock are required to spawn simultaneously. If this can be accomplished at regular and planned intervals throughout the year, research can be accelerated and commercial hatchery production undertaken to provide juveniles to on-growers at the best time for growth and market opportunities.

KGW are annual spawners, spawning between February and July in South Australia (Scott 1954, Bruce 1989, Cockrum and Jones 1992 as cited in Fowler and McGarvey 1997). Therefore, to achieve controlled egg production year round, spawning must occur within and outside the natural spawning season through environmental manipulation (i.e. phase-shifting the temperature and photoperiod to promote out of season spawning).

Natural spawning (release of eggs voluntarily without induction) in captivity, during both the natural and manipulated seasons, has been observed in some finfish species (Tucker, 1998). Natural spawning of fish at a predetermined period is the preferred method of spawning for both research and commercial purposes. This form of spawning can produce high quality eggs - has minimal impact on animals and reduces stress. It also eliminates the need for induction hormones and handling procedures, which will consequently reduce costs.

The use of induction hormones can be useful in species that do not undertake natural spawning in captivity or to achieve a higher spawning fraction (number of broodstock successfully spawning) at a known time. There are a variety of types and levels of induction hormones available. Previous success with snapper (*Pagrus auratus)* broodstock at SAASC was achieved using lutenising hormone releasing hormone, LHRHa ([des-gly^{10}, D-trp^{6}, pro-ethylaminde], a synthetic analogue of gonadotropin releasing hormone (GnRH)) administered as a slow release cholesterol/cellulose pellet at 25µg/kg body weight (bw). Other types of inducing hormones (i.e. Ovaprim®) are also available for fish induction. Ovaprim® contains

both a synthetic analogue of salmon gonadotropin releasing hormone (SgnRHa 20μg) and a dopamine antagonist (Domperidone 10mg/mL), which inhibits the release of gonadotropin releasing hormone (GnRH). The use of a dopamine antagonist can enhance the effect of GnRH in some fish species by reducing this inhibition / control pathway (Bromage and Roberts, 1995).

Current hormone induction methods however, only bring about final ripening of gametes and are of little use in radically altering the timing of maturation (Bromage and Roberts, 1995). To alter the timing of the natural spawning season, environmental manipulation (phase-shifting the temperature and photoperiod to promote out-of-season spawning) is required. Environmental manipulation allows commercial hatcheries to produce several hatchery runs a year and to plan production to suit market demands. A combination of environmental manipulation and hormone induction is often required to achieve out-of-season spawning.

The quality of eggs should also be examined when considering the potential of a species for commercialisation. Broodstock nutrition prior to spawning is one factor known to have an effect on the quality and quantity of eggs in most finfish (Watanabe *et al* 1984a; Watanabe 1985, Watanabe *et al* 1991a, b; Verakuniriya *et al* 1996; Izquierdo *et al* 2001). The level and type of hormone administered, the form of spawning (i.e. voluntary release of eggs, strip spawning) and the latency period (time between induction and spawning) may also affect the quality of spawnings. The effect of these factors needs to be considered when investigating the spawning and larval rearing of KGW.

Throughout this project, trials were conducted to investigate the natural and induced spawning of KGW broodstock during and out of the natural spawning season. An understanding of how KGW broodstock respond to various forms and levels of induction are fundamental in achieving successful egg production and furthermore aquaculture production of this species. If further domestication continues it may be possible to achieve a consistent supply of eggs from voluntary spawning of broodstock.

1.1.1 Investigating the natural spawning of KGW

Aim 1

To investigate the voluntary spawning of KGW broodstock (without induction) during the natural spawning season over a period of 3 years.

Methods

KGW broodstock were captured from the northern coast of Kangaroo Island, south of Adelaide, South Australia during May 1997. A total of 534 mature fish (average weight 370g) were captured and transferred to culture facilities at SARDI Aquatic Sciences. Upon arrival, broodstock were divided between two-40,000L flow-through outdoor tanks. Broodstock were held in these tanks for approximately 12 months until the next spawning season (February-July 1998).

One month before the anticipated beginning of the spawning season the stage of reproductive development of females was assessed using an internal endometrial biopsy to obtain an oocyte sample. A small amount of abdominal pressure was applied to males to assess if they had running milt. The majority of female broodstock (>70%) had vitellogenic, yolked oocytes and >60% of males had freely running milt.

One 40,000L tank of KGW broodstock were fed daily and left without any further interruptions (n=106 females; n=87 males). Fish in the remaining tank were implanted with LHRHa slow-release pellets at 25µg/kg (1.1.2 Induction of KGW broodstock with LHRHa pellets at 25µg/kg bw). Fish numbers varied throughout the years due to mortality (largely attributed to the disease outbreak, *Exophiala salmonis*) and the removal or addition of fish required for trials.

An egg collector was connected to the tank and inspected each morning. The total number of floating and sunken eggs was recorded.

Results/discussion

The duration of the first spawning season (1998) for broodstock after capture was shorter than is reported in the wild (generally extends from February to July (Scott 1954, Bruce, 1989, Cockrum and Jones 1992 as cited in Fowler and McGarvey 1997), with periodic egg production occuring from 14 April (1998) until 28 May (1998). Total egg production was

very limited, ~735,000 eggs (fertile and non fertile over a period of 44 days; n=106 females; n=87 males) with a low fertilisation rate (17.69%) (Figure 1.1a). Few females spawned, and those that did appeared to be experiencing problems synchronising with males, as indicated by the low fertilisation rate. Males produced milt from April until June (1998).

During spawning of captive broodstock, the second year after capture (1999), the spawning period was similar to the reported wild spawning season with eggs collected from 6 April (1999) until 8 June (1999), with the peak in late April - early May (Figure 1.1b). Egg production increased substantially but the numbers of eggs produced was not sufficient for commercial production (Figure 1.1). Average daily egg production was 59,505 (both fertile and non fertile over a period of 63 days; n=38 females; n=33 males) with a total of ~3,750,000, eggs collected for the spawning season. The fertilisation rate of eggs increased, from the previous spawning period, to 55.32%. The estimated spawning fraction (percentage of females spawning at one time) (based on fecundity estimates for KGW from Kangaroo Island with total length (mm) = 440; Fowler *et al*, 1999) increased this year up to 16.5%. Males produced milt from early April to late June 1999, which was similar to the previous year (1998).

It was thought that the spawning fraction and fertilisation rate would increase with further domestication. Results from the third spawning season (2000) demonstrated that this was not the case (Figure 1.1c). This suggests that natural spawning of wild-caught domesticated broodstock without induction cannot be relied upon to produce eggs for a commercial hatchery. The fertilisation rate of eggs spawned during the 2000 spawning season was 44.16% with the spawning fraction reduced to 7.6%. Average daily egg production was 71,869 (both fertile and non-fertile eggs over a period of 50 days; n=82 females; n=60 males) with a total of ~3,600,000 eggs collected for the spawning season (Figure 1.1c). The spawning period was shorter than the 1999 season occurring from 13 April (2000) until 2 June (2000) (a total of 51 days). Males produced milt from early April to mid-June. It seems likely that the compressed spawning season in 2000 can be attributed to higher than normal temperatures in March–April (seawater temperature, 20.9°C on 13/04/00) delaying the onset of spawning, and an unusually cold spike on the 28th May (seawater temperature, 14.1°C), causing spawning to end. This notion is in line with the previous spawning season results (1999), where spawning began and ended when the seawater temperature was 19.1°C and 14.8°C respectively. These results suggest that KGW broodstock require seawater temperatures between about 19°C and 15°C for spawning.

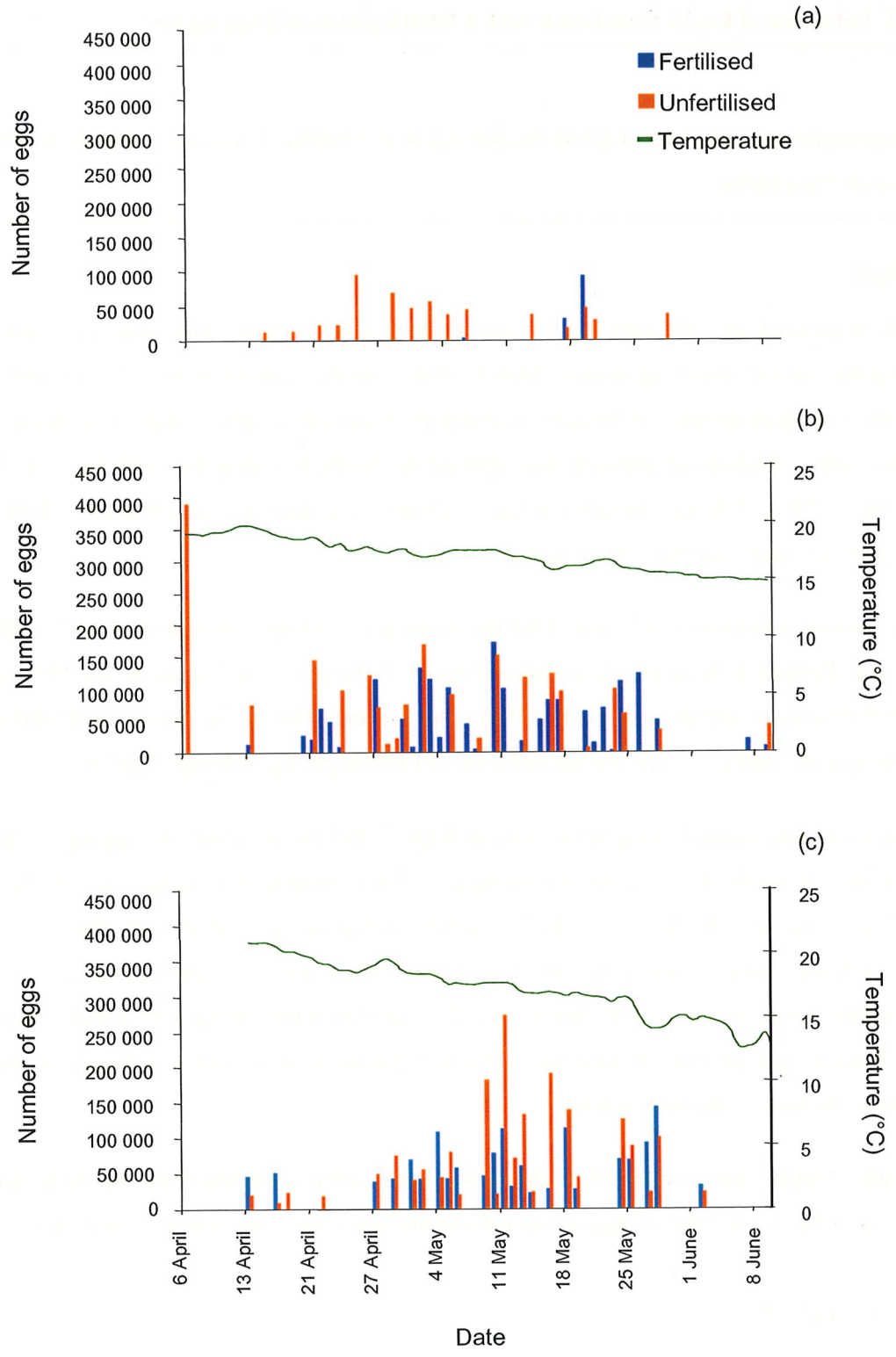

Figure 1.1 Voluntary egg productions from captive KGW broodstock during the natural spawning season of (a) the first (1998), (b) second (1999) and (c) third (2000) year after capture.

1.1.2 Induction of KGW broodstock with LHRHa pellets at 25µg/kg bw

Aim

To investigate the induction of KGW broodstock with LHRHa cholesterol/cellulose pellets at a dose of 25µg/kg bw.

Methods

KGW broodstock (n= 40) were held in one 40,000L outdoor tank. One year after capture, during the natural spawning season (March 1998), oocyte samples were collected and the reproductive development of females assessed from oocyte samples taken by endometrial biopsy tube. Abdominal pressure was applied to males to check for running milt. The majority (>70%) of female broodstock had vitellogenic, yolked oocytes (diameter >400µm) and >60% of males had freely running milt.

Slow release cholesterol/cellulose LHRHa ([des-gly^{10}, D-trp^{6}, pro-ethylaminde] LHRH analogue, Peptech Ltd) pellets were prepared based on the procedure by Lee *et al* (1985) with pellet formulation altered to provide 10% copha binder. The concentration of LHRHa in pellets was adjusted to contain the desired dose of LHRHa per kg in 40mg of pellet.

Broodstock were sedated using benzocaine at 40ppm. Pellets at a dose of 25µg/kg bw were implanted into the thick muscle behind the head, mid-way between the lateral line and slightly anterior to the first dorsal spine. Pellets were inserted using a pellet implanter into the anterior dorso-lateral musculature until the skeletal frame was felt (approximately 2-3cm). The pellet implanter was constructed from 2-210 stainless steel tubing with wooden handle drilled out to load pellets. A wire rod (1.5mm in diameter) was used as a plunger to push pellets to the base of the hollow tube.

An egg collector was connected to the tank. Eggs were collected from the tanks each morning. The total number of eggs collected and the fertilisation rate was recorded daily.

Results/discussion

Swelling of the gonadal region occurred 6-7 days following implantation in some KGW broodstock suggesting that gonadal development was occurring. After a period of 10 days (the time period at which snapper, *Pagrus auratus* released eggs following induction of LHRHa cholesterol/cellulose pellets of the same dose (Hutchinson, pers. comm.)) some

broodstock had become very swollen but no eggs had been released. 2 mortalities occurred 14 days after implantation. Mortalities had a very swollen abdomen with clumped eggs protruding from the gonadal pore, suggesting that some broodstock had become egg bound. No eggs were produced from broodstock.

These results indicate that although a dose rate of 25µg/kg bw of LHRHa was successful in promoting maturation in KGW broodstock, it failed to promote spawning (the release of eggs). Lowered doses of LHRHa, as a slow release pellet, may be more suitable to promoting the final stages of maturation and spawning in KGW. Doses of 25µg/kg bw of LHRHa followed by manual stripping of eggs may also be a feasible option, although this method is more labour intensive and may inflict more stress on the animal.

Alternative methods and doses of hormone induction need to be investigated for KGW. Spawning research during this project concentrated on alternative methods of promoting spawning, focussing around environmental manipulation, with and without low-level hormonal stimulation.

1.1.3 Environmental manipulation of KGW and investigation of low level LHRHa cholesterol/cellulose pellets as a priming pellet and different levels of LHRHa cholesterol/cellulose pellets as a follow up implant.

Aim 1

To induce captive KGW broodstock to undergo reproductive development outside of the natural spawning season using environmental manipulation (phase shifting the temperature and photoperiod).

Aim 2

To examine the effect of a priming pellet (LHRHa cholesterol/cellulose pellet at 10µg/kg) and varied doses of LHRHa as a slow release resolving pellet, on the spawning (egg quality and quantity) of KGW broodstock

Methods

Mature KGW previously housed in outdoor tanks were stocked in June 1998 into 4 x 10,000L tanks (n=45 per tank) supplied with recirculating seawater within a controlled environment facility. The water temperature and photoperiod were adjusted (Figure 1.2) to condense seasonal variations in an attempt promote spawning in November - 6 months in advance of the natural spawning season. Fish were assessed on 16 November 1998, at this time no reproductive development beyond primary oocyte stages was observed in females and no males with freely running milt were found. A decision was made to use this opportunity to investigate if oocyte development could be promoted using a series of hormone implants.

A combination of two intramuscular implants (LHRHa cholesterol/cellulose pellets) were administered at 3-week intervals to males (n = 20) and females (n = 25) in each treatment, in an attempt to promote reproductive development under a constant environment approximating that present at the natural spawning peak (May). As there was uncertainty as to the effectiveness of this approach, each treatment (tank) was implanted three weeks apart so that progress could be assessed. Initially a dose of 10 µg LHRHa/kg bw was administered to each treatment and the control as a priming dose. The dose rate of the second implant was 10, 15 and 20 µg LHRHa/kg bw for treatments 1, 2 and 3 respectively, with one tank serving as a control group (untreated). Pellets (90% cholesterol) were prepared following the method described by Lee *et al* (1986).

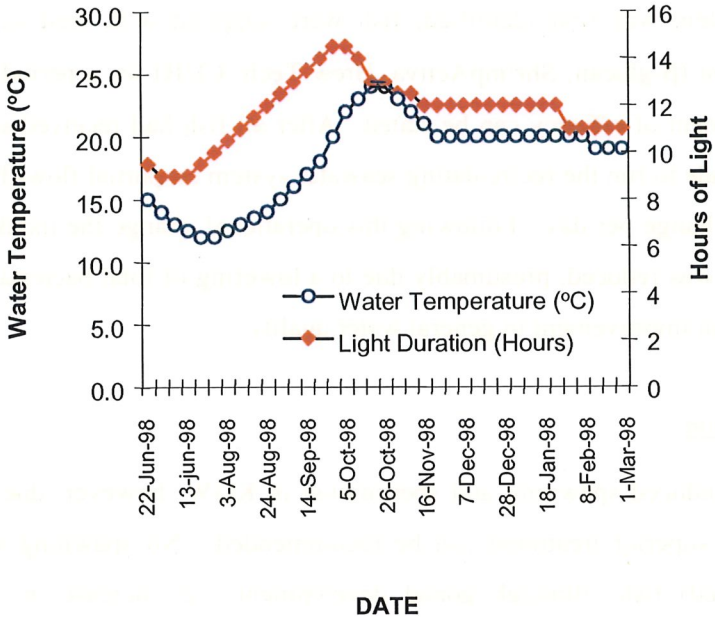

Figure 1.2 Environmental manipulation for controlled environment room housing KGW broodfish (June 1998 - March 1999).

Throughout the conditioning period (June - November), ongoing low-level mortality (1-3 fish per week) was observed for fish within the controlled environment facility and was attributed to the fungal pathogen *Exophiala salmonis*. It is suggested that the stress associated with this disease may have compromised the attempt to promote reproductive development through environmental manipulation.

At the time of each implant an ovarian biopsy sample was taken to stage reproductive development and determine the mean diameter of the 10 largest oocytes measured (n = 20 largest). After implantation, fish were left to spawn in the tank and egg collectors connected to the tank outlets were checked each morning and evening.

Following each treatment most fish developed ulceration at the location where the implant was administered, this often resulted in mortality or the need to euthanase effected fish. *Pseudomonas aeruginosa* and *Vibrio alginolyticus* were identified from samples submitted for pathology testing. These infections were presumed to be opportunistic and it is suggested that the stress associated with this level of infection would also have compromised results as, although eggs were induced, the numbers were low (Figure 1.3) and fertilisation rates were negligible.

When the problem was first identified, fish were supplied with feed supplemented with immunostimulant (β-glucan, ShrimpActiva, Brew Tech, CUB) for a period of 3-weeks. No accurate assessment of efficacy can be stated. After all fish had received second implants a decision was made to run the recirculating seawater system on partial flow-through to provide 100% water exchange per day. Following this operational change the incidence of infection of implant sites was reduced, presumably due to a lowering of total bacterial numbers within the system and an improvement in general water quality.

Results /discussion

All treatments induced spawning and spermiation in KGW, however, due to the generally poor results no superior treatment can be recommended. No spawning was recorded for control (untreated) fish, although gonad development (i.e. increase in oocyte diameter) progressed over the 11-week period, during which a constant environment was maintained (Figure 1.4). Oocyte development was accelerated by all hormone implant treatments (Figure 1.4). No spawned eggs were fertilised and the number of eggs collected was low, varying from 2,000 to 54,600 per spawn (Figure 1.3).

It is suggested that results of this trial were compromised by the incidence of the fungal pathogen *Expophiala salmonis,* a unique problem for KGW (1.2 *Exophiala salmonis* infection in captured KGW). Following implantation further infection by opportunistic bacteria (i.e. *Psueudomonas sp.* and *Vibrio sp.*) increased stress on broodfish. Regardless of this, all treatments induced ovulation and spawning of eggs and advanced the reproductive development of captive broodfish maintained under controlled environmental conditions.

To overcome difficulties observed, all tanks were de-stocked and the system disinfected before restocking with new wild caught fish (~100 KGW broodstock with a mean weight of ~300g, captured in September 1999, off the coast from Port Victoria, South Australia). In the future the addition of ozonation or UV filtration will be used to reduce bacterial numbers within the system when operating on complete recirculation, although satisfactory water quality can be maintained by incorporating a small constant water exchange amounting to 100% change per day.

Although no fertile eggs were collected, oocyte development (Figure 1.4) was promoted by all hormone treatments. The possible use of hormone pellet implants to accelerate reproductive development was demonstrated. Disease eradication and improved water

disinfection is recommended if controlled out of season production of high quality eggs is to be achieved.

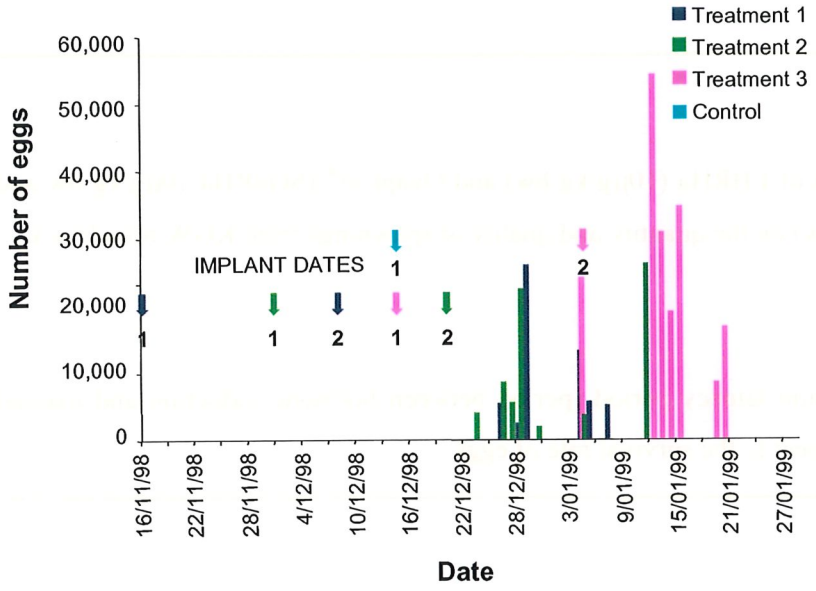

Figure 1.3 Egg production from KGW in treatment 1 (10µg/bw LHRHa priming and follow up pellet), treatment 2 (10µg/bw LHRHa priming pellet and 15µg/bw follow-up pellet), treatment 3 (10µg/bw LHRHa priming pellet and 20µg/bw follow-up pellet) and control (10µg/bw LHRHa priming pellet only)

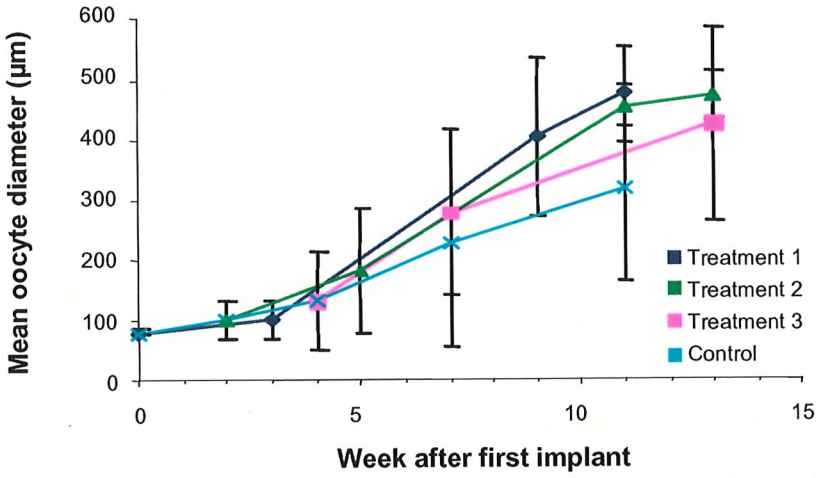

Figure 1.4 Mean oocyte diameters in treatment 1 (10µg/bw LHRHa priming and follow up pellet), treatment 2 (10µg/bw LHRHa priming pellet and 15µg/bw follow-up pellet), treatment 3 (10µg/bw LHRHa priming pellet and 20µg/bw follow-up pellet) and control (10µg/bw LHRHa priming pellet only).

1.1.4 Comparison of the effect of LHRHa (10µg/kg bw) and Ovaprim® (SGnRHa 10µg/kg bw and Domperidone 5mg/kg bw) as an injection on the reproductive development of KGW

Aim 1

To investigate the effect of LHRHa (10µg/kg bw) and Ovaprim® (SGnRHa 10µg/kg bw and Domperidone 5mg/kg bw) on the quantity and quality of spawnings from KGW broodstock.

Aim 2

To determine the optimum latency period (period between hormone induction and manual strip spawning) with respect to the survival rate of eggs.

Methods

First generation (F1) KGW (n=35; females=16 (mean weight = 396g); males=19; (mean weight=356g)) cultured at SARDI Aquatic Sciences during the 1998-spawning season were housed in a 40,000L flow-through outdoor tank. During the natural spawning season (May 2000) oocyte samples were collected from female broodstock by endometrial biopsy tube to determine stage of maturity. Abdominal pressure was applied to males to check if they were freely expressing milt. All females possessed vitellogenic oocytes (diameter > 400µm) and 100% of the males were freely expressing milt.

An experimental system of 8 x 500L flow-through tanks were set up to maintain treated broodstock. Three hormone induction treatments were established:

1. LHRH ([des-gly^{10}, D-trp^{6}, pro-ethylaminde] analogue (Peptech Ltd)) at 10µg/kg bw as an injection.

2. Ovaprim® injectable solution (SGnRHa 20µg/mL and Domperidone 10mg/mL, Aquatic Diagnostic Services International Pty Ltd) at 0.5mL/kg bw.

3. Control (injectable saline – 0.9%).

Three replicate tanks were randomly allocated for each treatment (n=4/replicate). However, due to limited space available only 2 tanks were allocated to the control treatment.

Broodstock were sedated using benzocaine (40ppm). The hormone treatments were administered into the peritoneal cavity of the fish. Fish were randomly allocated between the tanks depending on the treatment they received. Tanks were covered with shade cloth to minimise disturbance and left for 50 hours.

At 50 hours post-injection, females were removed from the tanks. Manual stripping of eggs was attempted for each fish. If eggs were not being expressed freely, fish were placed back into their respective tanks. Milt was collected from males to fertilise eggs collected from females in the same tank. Eggs were fertilised using the dry method of fertilisation (Tucker, 1998). Fertilised eggs were placed into 160L flow through egg incubators. Hatching success rates were recorded for each batch of eggs.

Fish were checked at 54 and 70 hours post injection to see if any females were freely releasing eggs, following the same procedure outlined above.

Results/discussion

In total 5 fish released eggs across all treatments. Due to the limited number of fish, no superior induction hormone can be identified for KGW spawning induction. No fish in the control treatment released eggs.

The number of females that released eggs in LHRHa and Ovaprim® treatments (3 and 2 respectively) were comparable as was the average hatching success rates in each treatment (28.06% and 37.89% respectively) (Figure 1.5 and 1.6). While it is hard to extrapolate from these results when so few animals were able to be strip spawned, it does suggest that neither of the treatments investigated in this trial is substantially superior to the other.

Hormone induction with Ovaprim® (10µg/kg bw) may have altered the rate of oocyte maturation with a female able to be stripped at 50 hours post injection (Figure 1.6). However, with a sample size of one, no conclusion can be made.

In both treatments there is a reduction in the quality of eggs stripped (hatching success) as the latency period (hours post injection) increased from 50 to 70. Disregarding treatment, the average hatching success rates for eggs stripped at 50, 54 and 70 hours post injection were 75.78%, 42.09% and 0% respectively. These results are limited due to the small sample size. However, they suggest that stripping fish between 50-54 hours post injection may optimise egg quality in KGW, within the latency periods investigated in this study.

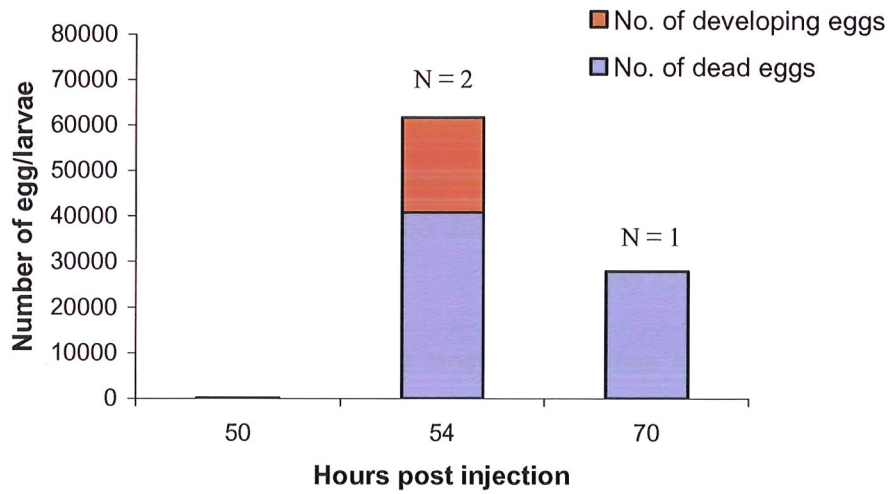

Figure 1.5 Number of larvae and dead eggs stripped from KGW broodstock injected with LHRHa (10µg/kg) 50, 54 and 70 hours post injection.

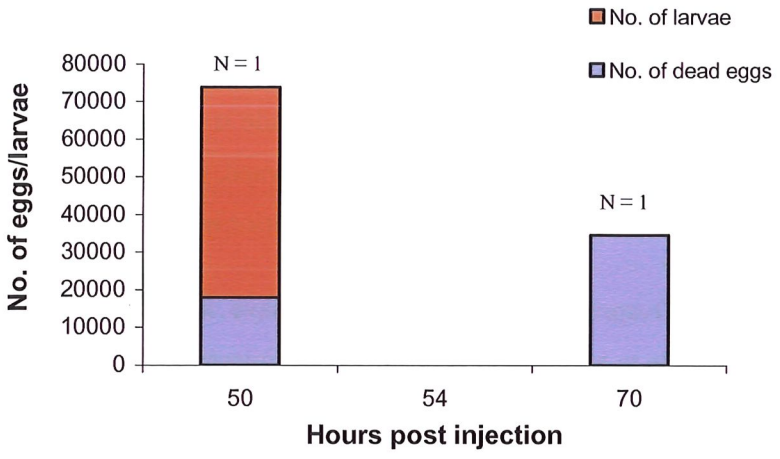

Figure 1.6 Number of larvae and dead eggs stripped from KGW broodstock injected with Ovaprim® (10µg/kg) 50, 54 and 70 hours post injection.

1.1.5 Investigation of LHRHa at 15µg/kg bw and Ovaprim® (20µg/kg bw SGnRHa and Domperidone 10mg/kg bw) on the reproductive performance of KGW

Aim

To examine the effect of LHRHa (15µg/kg bw) and Ovaprim® (20µg/kg bw SGnRHa and Domperidone 10mg/kg bw) as an injection on the spawning fraction and quality of egg produced in captive KGW.

Method

A total of 74 KGW broodstock (average weight 811g) were housed in a 40,000L outdoor flow through tank at SAASC for a period of 4 years. 39 fish had been previously tagged using a T-bar anchor tag, 35 fish remained un-tagged.

Three treatments were established (n=13/treatment):

1. LHRHa ([des-gly^{10}, D-trp^{6}, pro-ethylaminde] LHRH analogue, Peptech Ltd) at 15µg/kg bw.
2. Ovaprim® injectable solution (SGnRHa 20µg/mL and Domperidone 10mg/mL, Aquatic Diagnostic Services international Pty Ltd) at 1mL/kg bw.
3. Control (0.9% saline).

This trial was conducted in an attempt to identify an effective dosage for the induction of KGW, not as a comparison between LHRHa and Ovaprim®. Dosages of each treatment were based upon results of previous trials, in which 25µg/kg was considered to be excessive and the recommended dosage of Ovaprim® (10mg/kg bw SnRH and Domperidone 5 mg/kg bw) was found to be insufficient.

Broodstock were sedated using benzocaine (40ppm), their sex, weight and tag colour was recorded for tagged fish. Tagged fish were then randomly allocated a treatment and injected into the peritoneal cavity with LHRHa (15µg/kg bw), Ovaprim® (20µg/kg bw) or saline. Fish were then returned to the tank. Un-tagged fish were injected into the peritoneal cavity with LHRHa (15µg/kg bw) and returned to the tank.

At 52 hours post-injection, fish from all treatments that had ovulated, and were running ripe, were sedated and abdominal pressure was applied to females to determine if they were freely expressing eggs. Fish that were releasing eggs were manually stripped into a dry container. Milt was collected from males and used to fertilise eggs using the dry method of fertilisation (Tucker, 1998). Fertilised eggs were placed into 200L flow through incubators. Hatching success rates were recorded 24 hours after hatching.

Results / discussion

In total, the spawning fraction (the percentage of fish able to be successfully strip-spawned) in the Ovaprim® treatment were 36.36% (4 fish), compared to 23.08% (3 fish) in LHRHa treatment and 8.3% (1 fish) in the control treatment. The low percentage of fish in the control group to undergo voluntary spawning is in line with results from previous trials, which suggests that KGW are not a species that will undergo significant voluntary spawning in captivity. There was little effect on spawning fraction of KGW between LHRHa at 15µg/kg bw and Ovaprim® at 20µg/kg bw.

Average hatching success rates in the LHRHa 15µg/kg bw; Ovaprim® 20µg/kg bw and control treatments were 68.82%, 50.69% and 31.74% respectively (Figure 1.7). Variation in the hatching success rates within treatments makes it difficult to investigate the differences in egg quality between treatments. The low hatching success rates across all treatments suggest that the quality of the broodstock was not optimal prior to induction. This is attributed to delays in supply of good quality pellet feed (commisioned from Barneveld Nutrition Pty Ltd using the new SARDI Australasian Experimental Stockfeed Extrusion Centre) prior to induced spawning, highlighting the importance of nutrition in achieving good quality eggs.

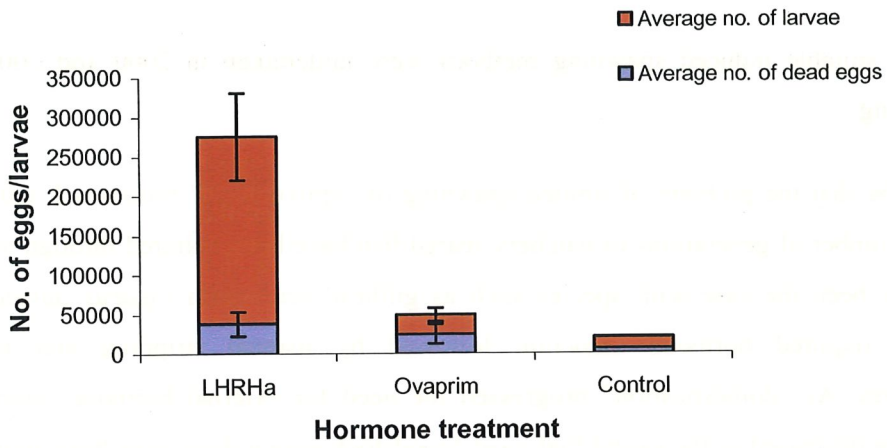

Figure 1.7 Average numbers of larvae and dead eggs produced in the 3 treatments (LHRHa, Ovaprim® and Control) (±SE).

Conclusion

Broodstock of many fish species, of interest to aquaculture, exhibit some degree of spawning dysfunction when maintained in captivity. This is most often observed as females completing vitellogenesis but failing to undergo final oocyte maturation (FOM), which precedes the release of eggs (i.e. spawning), and fertilisation (Mylonas and Zohar, 2001). Such spawning dysfunction probably results from the combination of chronic stress associated with captivity and the absence of appropriate environmental stimulus or cues. In these cases the use of hormone induced spawning methods is required.

Throughout this project, wild caught male KGW broodstock held in captivity completed spermatogenesis and were "running" throughout the spawning seasons (both natural and manipulated spawning seasons) while females completed vitellogenesis but few individuals progressed through FOM and spontaneous spawning was unreliable. During each natural season the spawning fraction (number of fish participating in spawning) was typically low (eg. 7.6% in the 2000 spawning season) with few eggs produced (eg. mean 72,000/day in the 2000 spawning season). In addition, the frequency of spawning was erratic and fertilisation rate varied between 0 % and 90% but was typically less than 50%. This scenario is another example of the most commonly observed type of spawning dysfunction in captive finfish broodstock where FOM is unpredictable or absent (Mylonas and Zohar, 2001). These factors together suggest that, for this species, greater control over egg production (i.e. timing, quantity and quality) is required for a commercial hatchery operation. Hence investigations to

develop suitable induced spawning methods were undertaken in 2000 and 2001 and are continuing.

It may be that the problem of limited spawning of captive KGW broodstock will diminish after a number of generations of hatchery reared fish have been cultured through to maturity. This has been the case with species such as gilthead sea bream *(Sparus auratus),* which initially required hormone induction followed by manual stripping and fertilisation procedures. As "domestication" progressed the need for external hormone intervention in spawning decreased to the extent that most broodstock spawn daily over three-month season (Barbardo *et al,* 1997). A similar trend is emerging with the culture of striped bass (*Morone saxatilis*), with FOM being observed more often but hormone treatments still neccesary to induce and synchronise spawning (Mylonas and Zohar, 2001).

It appears that most reproductive dysfunction of captive finfish species, including KGW, may be attributed to failure of vitellogenic oocytes to proceed through FOM. This can be overcome by administering GnRH (i.e. LHRHa or Ovaprim®) to provide the hormone surge needed to stimulate release of other hormones required to complete FOM. Further research in this area is required to provide commercial quantities of good quality eggs from captive KGW broodstock.

Trials throughout this project have demonstrated that hormone-induction procedures using the hormones LHRHa and Ovaprim® are successful in advancing the reproductive development and inducing ovulation and spawning of captive broodfish during both natural and manipulated seasons. Preliminary investigations into the optimum hormone dose and latency period (for broodfish being induced via injection) have indicated that doses between 10-20µg/kg bw (for both LHRHa and Ovaprim®), with a latency period between 50-54 hours, are most successful. However, the quality and quantity of eggs produced following this regime is not sufficient and further studies in these areas are required.

In addition investigation of first generation hatchery reared broodstock will continue to allow assessment of improved egg production over subsequent generations of domestication.

All studies investigating reproduction in KGW highlight the importance of broodstock nutrition. Diminished nutrition prior to spawning severely affects the quality and quantity of spawnings.

1.2 Exophiala salmonis infection in captured KGW, Sillaginodes punctata

Aim

To document health issues arising during the captive spawning of KGW.

Introduction

During this project a fungal disease *(Exophiala salmonis)* not previously reported in KGW, was detected. The disease outbreak was thought to compromise trial results (1.1.3 Environmental manipulation of KGW and investigation of low level LHRHa cholesterol/cellulose pellets as a priming pellet and different levels of LHRHa cholesterol/cellulose pellets as a follow up implant) and resulted in the de-stocking of a broodstock facility. Samples from fish infected with *Exophiala salmonis* also had *Pseudomonas aeruginosa* and *Vibrio alginolyticus* present. These infections were suggested to be opportunistic.

The results of this outbreak were documented in a paper by Reuter *et al* (below), which has been published in the Bulletin of the European Association of Fish Pathologists, 23 (3) 2003, 130.

Exophiala sp. infection in captured King George whiting (*Sillaginodes punctata*)

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Abstract

King George whiting captured in South Australia and placed in seawater tanks as broodstock developed ulceration of the skin and underlying muscle, particularly of the head, swim bladder and kidney, with a granulomatous inflammatory reaction evident microscopically. In special stains of the tissue, fungal elements were observed in the granulomas, and *Exophiala* sp. was isolated from affected tissue. The source of the infection could not be established. Similar agents have been responsible for outbreaks of clinical disease and mortality in farmed salmonids in the northern hemisphere, resulting in major economic problems for the fishery and aquaculture industries. This is the first report of the disease, involving a different species of fish, in the southern hemisphere.

Introduction

King George whiting, *Sillaginodes punctata*, is a popular marine finfish highly prized in South Australia by recreational and commercial fishermen and consumers. In 2000-01, commercial fishermen in South Australia caught 456 tonnes of King George whiting valued at $4.8 million from a total Australian catch estimated to be approximately 624 tonnes (ABARE, 2002). Most captured fish are in the size range of 30-40cm, although fish up to >50cm are taken from some regions. The majority of fish are <400g in weight (Fowler and McGarvey, 2000). Research has commenced on the suitability of this species for aquaculture, including development of methods to control egg production from captive broodstock, followed by larval rearing trials to determine procedures and optimal environmental conditions for culture of fingerlings. Initially broodfish, 370-600 mm in standard length, were captured from wild stocks. These fish were estimated to be 4 - 8 years old, based on the size and age of similar size fish in previous studies conducted in this area.

Exophiala species are black, "yeast-like" fungi that are commonly found in decaying wood, organic soil and associated watercourses (Ellis, 1994). Microscopically the thallus of the

fungus is composed of yeast-like cells when young, with hyphae and conidia developing within the culture over time. Clinical disease is occasionally reported in mammals, with cutaneous, disseminated or cerebral infections described in humans (Ziefer and Connor, 1986, Roncoroni and Smayevsky, 1988). Outbreaks of clinical disease due to *Exophiala salmonis* infection of farmed salmonids, such as Atlantic salmon (*Salmo salar*) and cut-throat trout (*S. clarkii*), have been seen in aquaculture operations in the northern hemisphere (Richards *et al,* 1978, Langvad *et al,* 1985, Ferguson, 1989). The disease generally presents as mortality, with granuloma formation in the brain or kidney. There have been occasional reports of similar disease in fish other than salmonids, such as a variety of aquarium fish (Blazer and Wolke, 1979). In Atlantic salmon held at a fish laboratory in Australia, lesions were seen on the head, lateral line and semicircular canals, with high mortality (Langdon and MacDonald, 1987).

Data on the pathology or microbial status of King George whiting in the wild or in captive situations is limited. This report describes the first documentation of an infectious agent affecting this species. It is also the first recorded occurrence of disease associated with *Exophiala* sp. as a cause of major economic concern to development of an aquaculture industry in the southern hemisphere.

Materials and Methods

Fish

Affected King George whiting originated from the northern coast of Kangaroo Island, south of Adelaide, South Australia. Approximately 550 mature fish (average 370 g) were hooked and maintained in a 200 L flow-through seawater tank on board a commercial fishing vessel. When 50-80 fish had been captured, these were transferred into two 7,000 L ballast tanks on board a large research vessel used to transport them to Port Adelaide. Fish were then transferred into a trailer mounted 2,000 L tank and stocked into a 40,000 L holding tank at the South Australian Aquatic Science Centre. Approximately 12 months after capture, 180 fish (45 fish per tank) were progressively stocked into four 10,000 L fibreglass tanks in a controlled environment room (ie. water temperature and photoperiod manipulation) operated using a recirculating seawater treatment system. Key components of this system are a rapid sand filter to provide mechanical filtration and a biological filter to remove ammonia. Following six months of culture in indoor tanks, all fish in tank C were anaesthetised with Benzocaine (P-aminobenzoate) prepared in 95% denatured ethanol at a dose of 60 ppm, and implanted with cholesterol/cellulose pellets containing LHRHa (des-gly$_{16}$, D-trp$_{6}$, pro$_{9}$-

ethylamide) at 10m/kg with 10% copha binder, to induce spawning. The pellet formulation and preparation was based on descriptions given by Lee *et al,* 1986. The pellets were implanted behind the head, mid-way between the lateral line and first dorsal spine, slightly anterior to the spine and approximately 2-3 cm into the anterior dorso-lateral musculature. Disease outbreaks occurred over the succeeding months in both indoor and outdoor tanks (Table 1.1). The frequency and severity of disease was higher in the controlled environment tanks. All indoor tanks were destocked due to persistence of the disease.

Pathology

Affected fish were examined and a complete necropsy was performed on selected samples. Portions of all major tissues and any lesions were fixed by immersion in 10% phosphate-saline buffered formalin. The fixed samples were processed and embedded in paraffin wax according to standard procedures. Sections (5 μm) of each tissue were stained with haematoxylin and eosin (H and E), Periodic acid Schiff (PAS), Gomori's methenamine silver (GMS), Gram and Ziehl-Neelsen acid fast (ZN) stains, and examined by light microscopy.

Microbiology

Portions of fresh lesions from the fish were inoculated onto horse blood agar, McConkey agar, Cholera TCBS agar and Sabouraud's agar containing supplementary chloramphenicol and gentamicin to inhibit bacterial growth. The plates were incubated in air with 10% CO_2 at 25°C for 10 days. (Agars were supplied by Oxoid, Basingstoke, U.K.). Plates were examined daily for visible colony formation. Individual bacterial colonies were identified by routine bacteriologic methods. Individual fungal colonies were identified by routine morphologic procedures at the Mycology Unit, Women's and Children's Hospital, North Adelaide. Suspect colonies were subcultured onto Sabouraud's agar and slide-cultures made of the resultant growth using potato-dextrose agar were examined after ten days. Fresh portions of ovarian tissue taken from live mature females during egg collection procedures, portions of fresh muscle from cockles (*Donax deltoides*) from batches used in the feed rations, swabbed samples of the sides of brooding tanks, samples of brooding tank sediment, and samples of sand from the water filtration system serving the tanks containing affected fish, were also cultured on the supplemented Sabouraud's agar.

Results

Gross Findings

The chronology of disease outbreaks in the holding tanks is described in Table 1. The initial outbreak occurred in an outdoor tank four months after capture of the animals. The first two fish died within one week, with deep oval ulcers, 5 to 8 cm long, extending from the dorsal surface of the head to the mouth (Figure 1.8). The underlying cranial bones were necrotic and partially destroyed, with soft white debris deposited on the surface of remaining portions. Circular raised white foci, 1 to 2 cm in diameter, were present on the skin of the side of the head. The internal organs appeared normal.

In the succeeding months, further fish were affected in both indoor and outdoor tanks. One fish showed problems with balance. At necropsy, deep skin ulcers and white necrotic patches were found on the ventral surface of the head between the gills, around the eyes and on the operculum. The grossly visible internal tissue damage was confined to white foci in the kidney in all but one fish. In this animal there was a broad area of tissue necrosis extending from within the abdominal cavity through the muscle beneath the spinal cord at the level of the posterior kidney. Within this area of tissue damage, the kidney contained multiple white foci of necrosis, 0.2 to 1.0cm in diameter.

Table 1.1 Timescale of disease outbreaks (suspected *Exophiala salmonis*) in outdoor and indoor tanks on site at SARDI Aquatic Sciences during 1997/98/99.

Date	Outdoor tanks	Controlled environment tanks
30/09/97	5 out of 230 broodstock in outdoor tank A, developed lesions affecting nasal areas. Suspected *Exophiala salmonis*.	
15/09/98	5 fish from outdoor tanks died over a 2 week period. All fish showed ulcerated lesions, suspected *Exophiala salmonis*.	
18/09/98		4 fish found with ulcerated lesions.
21/09/98	1 fish discovered floating upside down, using only pectoral fins to stay afloat.	
25/09/98		2 Infested broodstock exhibited loss of balance, using only pectoral fins to stay afloat. Fish at more advanced stages developed skin lesions.
06/10/98		Removed fish that showed any signs of disease, suspected *Exophiala salmonis*. 6 fish from a total of 4 tanks were removed.
07/10/98	All fish in outdoor tanks were checked for any sign of disease, none were found.	
11/11/98		One fish died in tank F, suspected *Exophiala salmonis*.
12/11/98	One fish died in tank A, haemorrhage occurring on dorsal side of fish.	
16/12/98		2 Fish in tank A were noticed to be ill. Skin discolouration, lethargic.
02/02/99		Disease still prevalent in tanks, all fish still showing signs of disease were removed. 10 fish in total were removed.

Eighteen months after capture, one of the 45 whiting in outdoor tank B exhibited skin haemorrhages around the anal opening, extending cranially along the ventral abdomen. There had been no previous disease incident in this tank. At necropsy, the abdomen contained serosanguinous fluid, with adhesions of intestine to the peritoneum around the cloaca. The swimbladder was distended, with a 1.0 cm thick wall and contained watery fluid. The inner lining was grey-green and covered with scattered white soft plaques. One month after this, one of 45 fish in tank C showed discolouration of the skin of the side of the head and reddening of the conjunctivae of both eyes, and another was lethargic with no visible skin lesions. At necropsy, both fish had pale livers and soft mottled kidneys, with occasional pale

foci of necrosis, 0.1 cm in diameter, in the renal parenchyma. The lining of the swimbladders was thickened to 0.2 cm. No other gross lesions were detected.

Histopathology

The microscopic changes in the skin, bone, swimbladder and kidney samples from affected fish were similar in all cases. There were multiple areas of parenchymal cell necrosis with infiltration of mononuclear inflammatory cells in the surrounding parenchyma and adjacent connective tissue, typical of granuloma formation in fish (Figure 1.9). There were faint outlines of structures resembling hyphae and other non-descript fungal elements seen in the granulomas in the H and E stained sections. These structures stained intensely with Gomori-methenamine silver stain and Periodic-acid-Schiff stain (Figure 1.10). Sections stained with Gram and Ziehl-Neelsen stains did not show any bacteria in the granulomas. Microscopic examination of all other internal organs, including brain, from all of the necropsied fish, failed to detect any lesions.

Microbiology

Bacteriologic culture of affected tissue samples of swimbladder, kidney, skin and bone yielded a moderate mixed growth including *Aeromonas hydrophila, Plesiomonas shigelloides, Vibrio alginolyticus* and *Pseudomonas* sp. Culture of the water samples, cockles and ovarian tissue from the live fish yielded only a light mixed growth of coliform bacteria. Culture of affected tissue samples of swimbladder, kidney, skin and bone on Saboraud's agar consistently yielded a moderate to heavy pure growth of a black fungus. Further identification was performed at the Mycology Unit, Women's and Children's Hospital, North Adelaide. The thallus of the fungus was black to dull olive-green, with a sharp margin. Grey aerial mycelia formed with age. When the slide culture was examined under the microscope, flask-shaped conidiogenous cells arose both terminally and laterally from thin-walled septate hyphae. Intercallary fertile cells were also present. The conidia were 0-3 septate, hyaline, thin-walled and ellipsoidal in shape, and were formed from scarred areas on the annellides from which vague annellations could be discerned. These features allowed the differentiation of this fungus from other species within the *Exophiala* genus. The characteristics were similar to those exhibited by *Exophiala salmonis.* Portions of ovarian tissue from mature females, portions of fresh feed ration ingredients, samples of brooding tank water, sides and sediment, and samples of sand from the water filtration system were negative for fungi on culture.

Figure 1.8 Deep skin ulcers on the head of a 4 year old King George whiting *(Sillaginodes punctata)*.

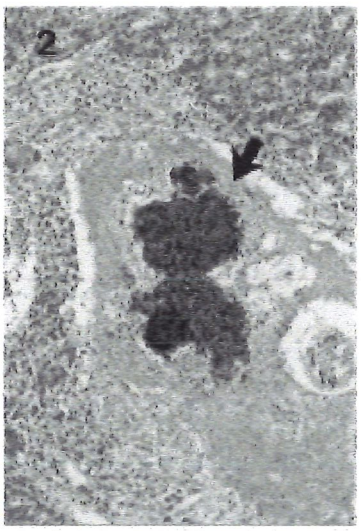
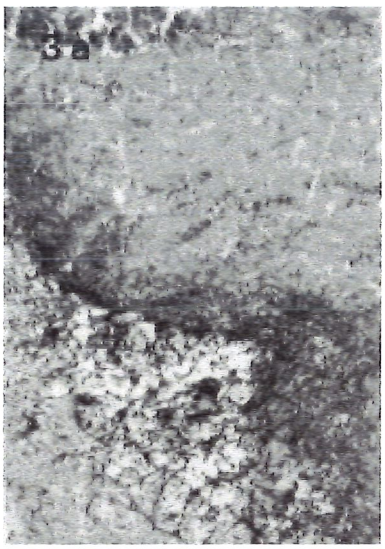


Figure 1.9 *Exophiala* infection in kidney from *S. punctata*. Area of necrosis containing hyphae surrounded by a granulomatous response (H and E X 200).



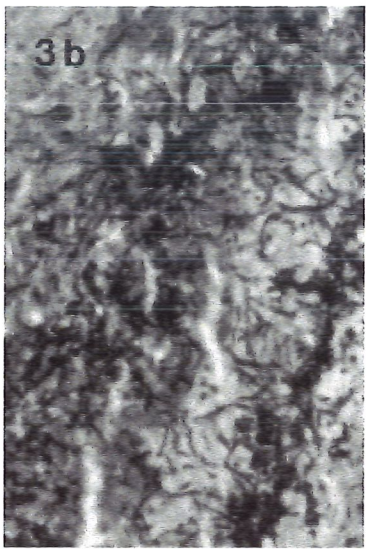


Figure 1.10 Photomicrographs of fungal elements. A. Swimbladder of *S. punctata* infected with *Exophiala* showing mats of hyphae surrounded by inflammatory cells and oedema (PAS X 200). B. Higher magnification showing variable morphology of fungal elements (GMS X 1000).

Discussion

In contrast to the situation in the whiting, in which skin, kidney and swimbladder were consistently affected, *Exophiala salmonis* infection and associated lesions in farmed trout and salmon have mainly involved the brain, eye, gill, kidney and head of fish less than 4 to 5 years of age (Carmichael, 1966, Langdon and McDonald, 1987). The reason for the altered tissue predilection and age of affected fish is not known. Predisposing factors could include different host family or fungal strain influence, different route of exposure, mode of transmission, or environmental conditions in the southern hemisphere. These factors were not examined in this study. There are several species present in the genus *Exophiala.* The organism in this case showed features similar to *Exophiala salmonis*. However, some of the individual strains are highly variable in character and specific identification can be difficult. The apparent difference in organs targeted in the King George whiting and the salmon and trout could be a reflection of strain variation between the agents isolated in these cases.

The exact source of the *Exophiala* sp. Infection was not identified. While these fungi are commonly found in soil and associated water samples, they were not detected in the immediate environment of the affected fish, although the sensitivity of the selective fungal culture was thought to be adequate to detect low levels of the organism. There was no evidence of transovarian transmission. It is possible that there was a sub-clinical skin infection in one or more of the captured stock placed in tank A, with subsequent clinical manifestation and transmission by fish-to-fish contact stimulated by the stressors of capture, transportation, handling, injection to produce spawning and higher stocking density.

The pathogenesis of the *Exophiala* sp. Infection in affected fish was not defined. It is considered likely that the infections commenced as small skin erosions, possibly related to abrasions or wounds, which developed to local ulcers and/or deep tissue infections. This pathogenesis has been suggested in *Exophiala* sp. Infections in other fish and in mammals. Infection of swimbladders is a common feature in disseminated infections by other fungi such as *Phoma herbarum*, *Saprolegnia* sp. and *Phialophora* sp. In contrast to the marked granulomatous response noted in the whiting in this study, localized necrosis and haemorrhage without a cellular response are usually seen in fungal infections of salmonids (Ferguson, 1989). This may reflect a variation in lymphoid cell response markers in whiting.

There was a clear association of the presence and isolation of the *Exophiala* sp. with clinical signs and lesions in the affected fish, with no such association in unaffected organs. The bacterial isolates obtained were not considered primary pathogens. This fungal infection may be a significant disease affecting mature King George whiting in aquaculture. If so, the

possibility of mortality and production losses during breeding and growth phases of this species will need to be considered. Food processing facilities will also need to be aware of the possibility of lesions due to this organism.

Conclusion

The outbreak of the fungal disease *Exophiala salmonis* and associated opportunistic bacteria was the only major health issue encountered during the captive spawning of KGW. No treatment for *Exophiala salmonis* was identified, however, early detection of the presence of the disease in fish stocks and de-stocking of infected fish controlled further outbreaks.

Acknowledgments

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Chapter 2 Broodstock: estimates of spawning frequency and periodicity

Introduction

KGW are the second most valuable finfish species in South Australia (Southern Fisheries, 1999). This species is heavily targeted in both the commercial and recreational sectors. The importance of this fishery lead to an FRDC funded study by Fowler (1995-1997) that focused on the reproductive biology of KGW, providing information to assist the development of effective management practices which are necessary to ensure the sustainability of the fishery (Fowler *et al*, 1999).

Fowler (1999), found that KGW are multiple batch spawners with asynchronous development and indeterminate fecundity. This has important implications for estimates of spawning biomass in fisheries models. Prior to studies by Fowler *et al* (1999), fecundity estimates of KGW were calculated from the standing stock of advanced vitellogenic (yolked) oocytes >300μm in diameter. However, as KGW are multiple batch spawners, numerous batches of oocytes are recruited from small pre-vitellogenic oocytes during the spawning season (Fowler *et al*, 1999). Therefore, the standing stock of advanced, yolked oocytes gives no indication of annual fecundity. To estimate spawning biomass using the Egg Production Method in species that employ this reproductive mode, it is necessary to have an estimate of both spawning frequency and batch fecundity made during a survey period (Hunter and Macewicz, 1985).

Two methods of estimating spawning frequency were trailed in this study. The first method (2.1 estimates of spawning frequency and periodicity by visual observation) used the degree of abdominal swelling in captive female broodstock to indicate a spawning event and the period between spawning events was used to estimate spawning periodicity. Scoring of broodstock was based on the degree of swelling in the abdominal area, which was used as an indication of the reproductive stage of the fish. This method assumes that a progression from stage 4 (very prominent swelling to the extent where muscle striations can be seen on the sides of the abdomen in the vicinity of the gonadal pore) to stage 1 (no visible development in the dorsal side (belly) of the fish) is the result of a spawning event. The validity of these results depends on whether spawning frequency in captive fish is a true representation of spawning frequency in the wild.

The second method trialed included assessment and documentation of the deterioration of post-ovulatory follicles (POF) within an ovary (2.2 Examination of KGW post-ovulatory follicles to assist with fisheries models) at known times after a spawning event. A POF is formed at ovulation when an hydrated oocyte is released from the follicle that previously surrounded it. At this stage the follicle remains intact inside the ovary and collapses away from the opening formed from the release of the hydrated oocyte (Hunter and Macewicz, 1985). The post-ovulatory follicle (POF) rapidly deteriorates and is reabsorbed. The presence of POF in an ovary is a positive indication of spawning and can be used to estimate the time since spawning and to determine spawning frequency. POF may also be useful to test hypotheses about events that trigger spawning and benefit larval transport (Taylor, 1984; Checkly *et al*, 1988 as cited in Fitzhugh and Hettler, 1995).

Hunter and Goldberg (1980) originally developed the method for using POF as an estimate of spawning incidence for the northern anchovy, *Engraulis mordax*.

To examine the degradation of POF, two main options are available. A field study can be conducted, capturing fish over a 24-hour period during the spawning season and estimating the time of spawning and degradation of POF accordingly. The second method involves induction of fish to spawn under laboratory conditions. Fish are then stripped so that an exact spawning time can be established. The latter method is more precise and is therefore the preferred method for documenting the degeneration of POF (Fitzhugh and Hettler, 1995).

2.1 Estimates of spawning frequency and periodicity by visual observation

Aim

To estimate spawning frequency in KGW broodstock by visual assessment of their reproductive development.

Methods

KGW broodstock were held in two 40,000L flow-through outdoor tanks at SARDI Aquatic Sciences. Female broodstock (n=25/tank) were tagged with T-bar anchor tags for identification. Tank 1 broodstock were left under natural conditions. Broodstock in tank 2 were implanted with LHRHa slow release pellets at 25µg/kg. Individual female broodstock were visually assessed daily during the spawning season (May-June) and attributed a score

(stage 1 to 4) based on the degree of swelling in the abdominal area of the fish (Table 2.1). The degree of swelling in the abdominal region is used as an indication of the reproductive stage of the fish, with stage 1 indicating little or no development and stage 4 indicating that the gonad of the female contains hydrated oocytes preceding a spawning event.

Data was collated in Microsoft Excel™.

Table 2.1 Reproductive development stages for KGW based on visual assessment of broodstock

Stage 1	No obvious swelling of the abdominal region.
Stage 2	Minor swelling anterior of the vent.
Stage 3	Obvious swelling of the abdominal region anterior of the vent.
Stage 4	Very prominent swelling to the extent that muscle striations can be seen on the abdomen in the vicinity of the vent.

Results/discussion

Monitoring the degree of swelling in the abdominal region of KGW broodstock on a daily basis was found to be an unsuitable method for estimating spawning frequency and periodicity in KGW. KGW, like many fish species, experience spawning dysfunction in captivity.

In this trial female broodstock from tank 1 appeared to undergo some degree of reproductive development in that they proceeded to stage 2 and 3. A limited percentage of females proceeded to stage 4 (Figure 2.1(A)). However, few progressed through final oocyte maturation (FOM) to spawning. As a consequence of this, it is not possible to estimate spawning frequency in tank 1 and use this as an estimate of spawning frequency and periodicity in the wild.

The percentage of females proceeding to stages 3 and 4 increased in tank 2 (Figure 2.1(B)), however, few females underwent spawning. This suggests that the LHRHa dose was sufficient to promote FOM but was not sufficient to promote spawning. Due to the use of LHRHa and the failure of broodstock to undergo spawning in captivity, spawning frequency

and periodicity in tank 2 cannot be estimated and is not considered a true representation of spawning frequency in the wild.

The second method (2.2 Examination of KGW post-ovulatory follicles to assist with fisheries models) was then trialed. This method assessed and documented the deterioration of POF within an ovary at known times after a spawning event.

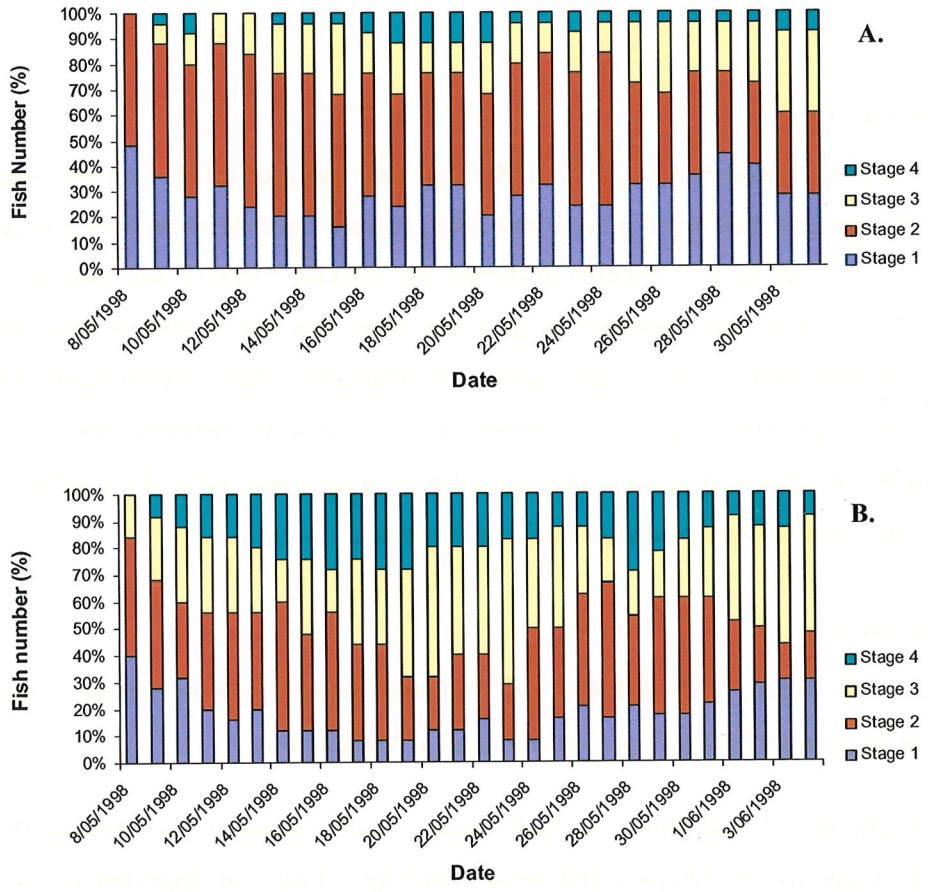


Figure 2.1 (A) Percentage of broodstock under natural conditions (i.e. no form of induction) at different reproductive stages based on visual assessment. (B) Percentage of broodstock implanted with LHRHa slow release cholesterol/cellulose pellets at 25µg/kg/bw at different reproductive stages based on visual assessment.

2.2 Examination of KGW post-ovulatory follicles to assist with fisheries models

Aim

To develop an accurate description of post-ovulatory follicles at know times after spawning to allow the spawning frequency of KGW to be estimated based on the stage of POF in the ovary.

Methods

Collection of broodstock

On 22 April 2001, KGW broodstock (n = 177; 178-735g) were captured off Pt.Victoria, South Australia. Once captured, fish were placed into 200L barrels supplied with flow through seawater. On arrival at the dock, fish were transferred into a 2000L fish tank and transported to the SAASC, where they were sedated using a low-level anaesthetic (benzocaine) and transferred to a 10,000L tank in a recirculating seawater treatment system. Temperature was maintained at 18.5°C as this is considered representative of seawater temperatures, in the wild, during the KGW spawning season. An egg collector was connected to the outflow of the tank so that any spontaneous spawnings could be recorded.

Start of the experiment

On 23 April 2001 (between 10am and 4pm) all fish were treated with an intraperitoneal injection of LHRHa (des-gly^{16}, D-trp^{6}, pro^{9}-ethylamide) at 10µg/kg bw.

On 25 April (between 12 and 6pm) all fish were visually assessed for reproductive development, as suggested by obvious abdominal swelling. Fish that appeared to have hydrated oocytes were strip-spawned if eggs could be released with moderate abdominal pressure. Fish that were successfully stripped (n = 18) were transferred into a holding corf within the tank. The time of spawning was recorded and either corf number or fin clipping identified fish. Milt was collected from male fish by expressing into a 2mL syringe. Any eggs collected were dry fertilised and incubated for future larval rearing trials.

Fish were sampled at 6-hour intervals (n=3) over a period of 36 hours. Sampling intervals were set at 6 hours as this was thought to capture major changes in the deteroriation of POF and could be achieved with the number of that responded to induction. The sampling period

was confined to 36 hours as previous studies (Hunter and Macewicz, 1985) suggest that POF structures are not easily identified beyond this period at the seawater temperatures examined in this study (18.5°C). Fish that had been stripped were euthanased by either spiking or an overdose of benzocaine. A single mortality (n=1) experienced at 3 hours post-spawning allowed one fish to be sampled at this time. Once euthanased, total length (mm) and weight (g) were recorded. The ovaries were then removed and weighed. A sample of the ovary was taken and stored in formalin acetic acid (FAAC) solution.

The fixed samples were then processed and embedded in paraffin wax according to standard procedures. Sections (5μm) were stained with haematoxylin and eosin (H and E) and examined by light microscopy.

It was found that 11 of the 18 fish sampled had hydrated oocytes in their ovaries at the time of sampling. Of the 11 fish that contained hydrated oocytes in their ovaries, 9 had ovulated at the time of sampling. Consequently, histological sections of ovaries from fish that had ovulated at the time of sampling contained different age POF structures (the POF structures that resulted from strip-spawning and other POF structures that resulted from subsequent ovulations post strip-spawning). This made it difficult to determine what the age of the POF structure was at the time of sampling.

As no eggs were found in the tank egg collector prior to strip spawning we can assume that no fish spawned in the period between injection and strip spawning.

It is clear from previous POF studies that, in temperate waters (13-19°C), POF structures become difficult to classify at 28 hours post-spawning and by 48 hours post-spawning are very easily confused with atretic follicles making it virtually impossible to identify (Hunter and Makewicz, 1985). As fish did not spawn in the 48 hour period between injection and strip spawning, and any POF structures that remained from a spawning prior to injection would have degenerated past the point of identification, it is reasonable to assume that the oldest POF structure in each ovary section is representative of that sampling time. Our results therefore consider the oldest POF in a single ovary to be associated with that sampling period.

Results/discussion

The degeneration of POF in most teleosts is thought to be similar to that of the northern anchovy *Engraulis mordax* (Hunter and Macewicz, 1985). Our results of KGW POF are in line with this, following the same general process.

The results of this trial are provided in 6-hour intervals up to 30 hours (6, 12, 18, 24, and 30) post strip spawning (below). POF structures were easily confused with atretic cells and connective tissue beyond 12 hours, and could not be accurately identified past 30 hours. A fish mortality at 3 hours post spawning provided a sample, however, due to a lack of replication at this sampling period the results at this time can not be considered representative of KGW in general.

3 hours post-spawning (Figures 2.2 a-c)

POF that appeared to have resulted from a spawning 3 hours previous, were quite numerous in the ovary. POF at this stage were typically irregular in shape, large in size (~260 x 130µm) with a large and distinct lumen. They have a convoluted or looped internal structure. The thecal layer (outer layer of cells) contains blood capillaries and is closely adhered to the granulosa layer. The granulosa cells are in an orderly linear arrangement. They are elongated with a prominent nucleus generally located at the apex of the cell.

6 hours post-spawning (Figures 2.3 a-c)

Degeneration of the POF is starting to become evident. The size of the POF is reducing (~190 x 80µm). The granulosa cells are starting to collapse into the centre of the POF and no longer maintain their orderly arrangement. As a consequence the POF is losing its convoluted/looped structure. The granulosa nuclei are spherical in shape and clearly evident. An obvious lumen is no longer present. A few vacuoles and pycnotic nuclei appear in the granulosa layer. The thecal layer is retaining its structure but is no longer adhered to the granulosa layer.

12 hours post-spawning (Figures 2.4 a-c)

Degeneration of the POF is now clearly visible. POF are further reduced in size (~150 x 60µm). At this stage the POF is becoming less irregular in shape and the cells have broken down to form a mass of cells. The nuclei of the granulosa cells are still visible, although the

shape and arrangement of cells cannot be distinguished. A few vacuoles are present in the granulosa layer. The thecal layer is still maintaining its intact structure.

18 hours post- spawning (Figures 2.5 a-c)

The POF is now greatly reduced in size (~110 x 50μm). It is now a compact, clumped structure. Granulosa nuclei are still visible within the structure although they have greatly reduced in number. At this stage the thecal layer is still intact although the first signs of deterioration are evident.

24 hours post-spawning (Figures 2.6 a-c)

The POF is now a very small clumped structure (~90 x 30μm) and is becoming hard to distinguish from connective tissue. Irregular shaped granulosa nuclei are evident. A few vacuoles are present. The thecal layer is no longer visible as granulosa and thecal cells have deteriorated into one mass.

30 hours post-spawning (Figures 2.7 a-c)

The POF structure is so small (~20 x 10μm) that it can only be identified on magnifications of 400x or higher. The cells have a clumped, almost triangular shape, at this stage. Very few irregular-shaped granulosa nuclei are visible. Small vacuoles may be present. There is no thecal layer at this stage. From this point, POF can no longer be accurately identified.

The results of this study provide a key of KGW POF degeneration from 3 hours to 30 hours post strip spawning. This key allows POF within a KGW ovary sample to be identified within a 6-hour period at 18.5°C. This information would be useful in estimating time from spawning, and consequently spawning frequency, in KGW. This reproductive information is fundamental to provide accurate estimates of spawning biomass in KGW, which will assist in developing effective management practices for the commercial fishery.

The finding that KGW continued to hydrate and ovulate after being strip-spawned at 48 hours post injection has implications for the induced spawning of KGW. The latency period (period between hormone induction and strip spawning) used in this study was 48 hours, based on previous trials. However, the discovery of hydrated females, continuing to ovulate up to 36 hours post strip spawning, indicates that 48 hours is not an ideal latency period for KGW. This result suggests that further trials are needed to examine in more detail, the optimum latency period with respect to controlled egg production using hormone-induction methods.

3 hours post spawning

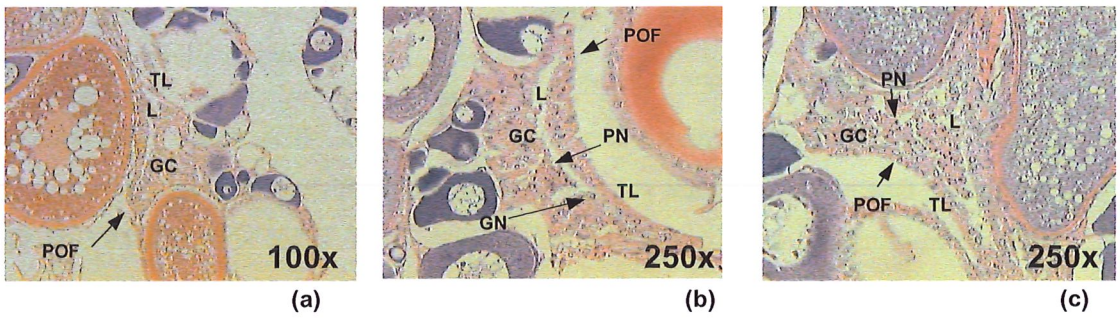


Figure 2.2 (a-c) KGW POF 3 hours post strip spawning.

6 hours post strip spawning

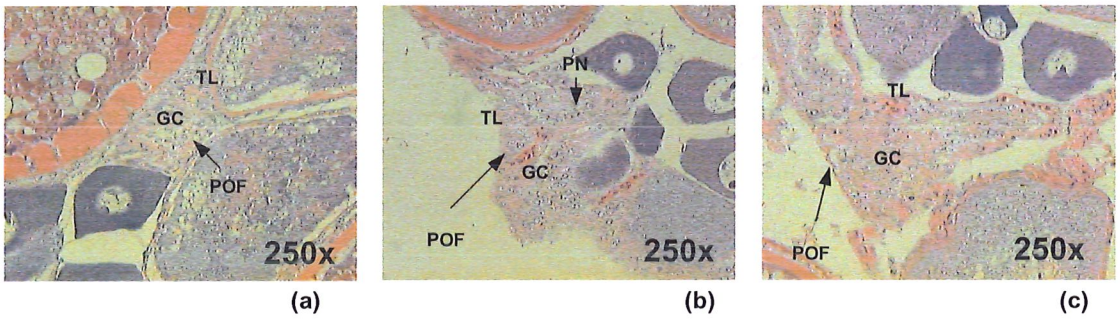


Figure 2.3 (a-c) KGW POF 6 hours post strip

12 hours post strip spawning

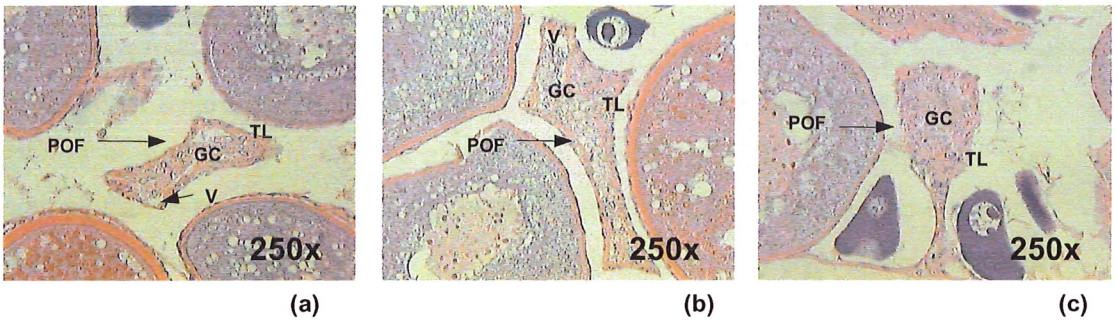


Figure 2.4 (a-c) KGW POF 12 hours post strip

POF = Post ovulatoryfollic ; GC = Granulosa cells; TL = Thecal layer; GN = Granulosa nucleus; L = Lumen; V = Vacuoles; PN =Pycnotic nucleus

18 hours post strip spawning

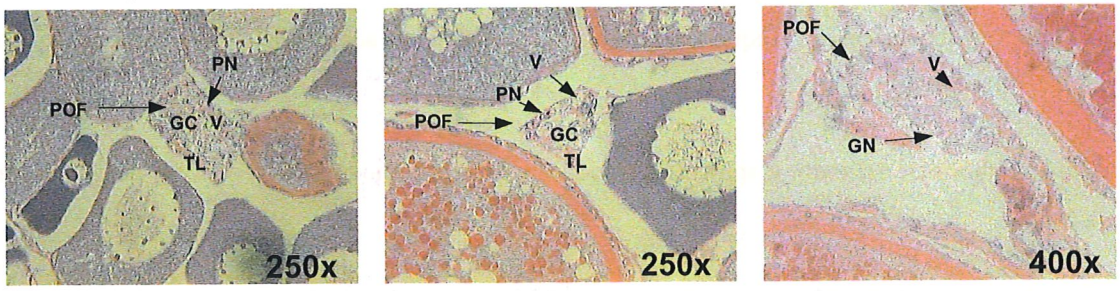


Figure 2.5 (a-c) KGW POF 18 hours post strip spawning.

24 hours post strip spawning

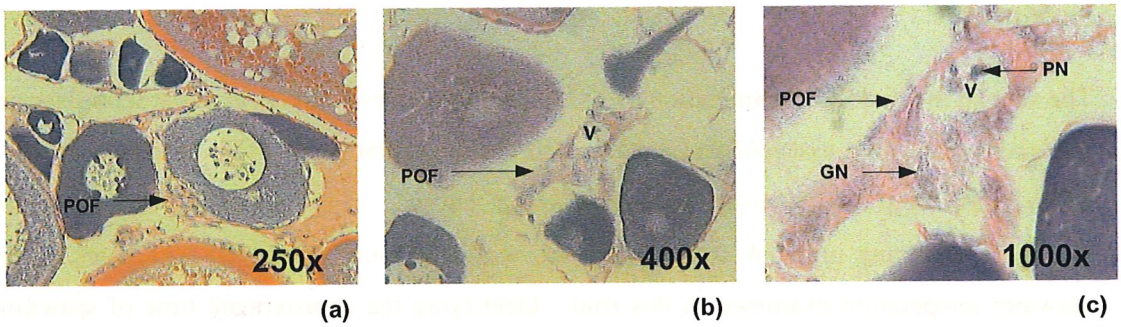


Figure 2.6 (a-c) KGW POF 24 hours post strip spawning.

30 hours post strip spawning

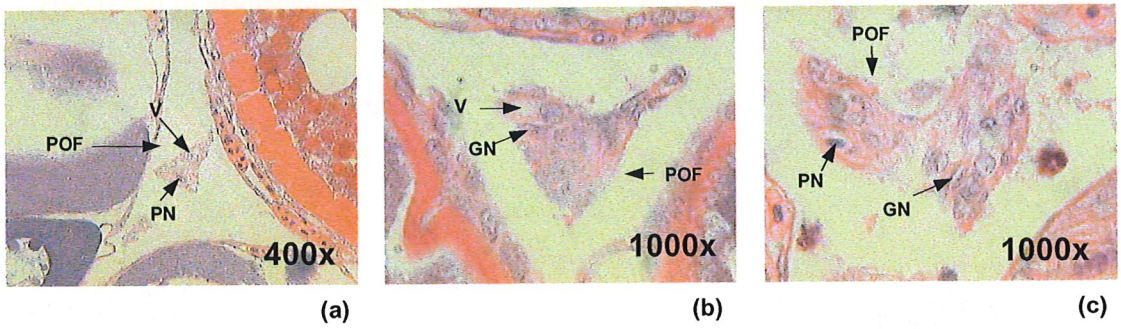


Figure 2.7 (a-c) KGW POF 30 hours post strip spawning.

POF = Post ovulatoryfollic ; GC = Granulosa cells; TC = Thecal layer; GN = Granulosa nucleu; L = Lumen; V = Vacuoles; PN = Pycnotic nucleus

Conclusion

Estimating the spawning frequency is required in KGW to accurately estimate fecundity for fisheries models.

Two methods of estimating spawning frequency and periodicity in KGW were trialed in this study.

The first method (2.1 Estimates of spawning frequency and periodicity by visual observation) was found to be an unsuitable method of estimating spawning frequency and periodicity in KGW, primarily due to the spawning dysfunction that is experienced in captive KGW broodstock.

The second method (2.2 Examination of KGW post-ovulatory follicles to assist with fisheries models) successfully assessed and documented the degeneration of POF structures within a KGW ovary from 3 to 30 hours post spawning at 18.5°C. This information will allow the timing of spawning in KGW to be estimated, within 6 hours, from POF structures, at the seawater temperature examined in this trial. Identifying the approximate time of spawning will allow spawning periodicity and frequency to be estimated and annual fecundity to be determined. The ability to determine annual fecundity will provide a more accurate method of calculating spawning biomass of KGW and hence assist in the management of this fishery.

Chapter 3 Larval Rearing

3.1 Egg development and hatching success rates

3.1.1 Oocyte development in KGW

Introduction

To achieve controlled spawning of captive broodstock it is necessary to understand the development of oocytes in KGW females. Induction hormones (e.g. LHRHa , HCG, CPE and Ovaprim®) can be effective in the spawning of finfish, however they only bring about the final stages of maturation of gametes and are of little use in radically altering the time of maturation (Bromage and Roberts, 1995). Before hormones can be administered it is necessary to ensure that vitellogenic oocytes are present. Samples of oocytes can be collected from the ovarian tissue of sedated female broodstock using a cannula or endometrial biopsy tube. Advanced stages of developing oocytes can be assessed visually or by binocular microscope. Histological sections can be prepared from oocyte samples and the internal structure of the oocytes examined under light microscopy. Use of microscopic and macroscopic views of oocytes enables the stage of oocytes to be compared and validated.

Aim

To construct a key documenting the development of KGW oocytes both macroscopically and microscopically.

Methods

Ovarian biopsy samples of KGW oocytes were taken from female broodstock progressively through their reproductive development. Females were sedated using benzocaine at 40ppm. An endometrial biopsy tube was inserted into the gonadal pore. The oocyte sample was removed and stored in 10% formalin in seawater.

Photographs were taken and the mean size of oocytes calculated at different stages using a image analysis system.

The fixed samples were processed, embedded in paraffin wax, sectioned to 5µm, stained with haematoxylin and eosin (H and E) and examined by light microscopy. Photographic images were taken.

A key (Figure 3.1) was constructed to compare the microscopic and macroscopic view of KGW oocytes at the four main developmental stages (primary oocyte (immature), pre-vitellogenic oocyte (unyolked), vitellogenic oocyte (yolked) and hydrated oocyte).

Results/discussion

Eggs are formed within folds of ovarian tissue of females. The stem-cell population of oogonia (germ cells destined to form eggs) undergo mitotic divisions to give rise to secondary oogonia. Secondary oogonia undergo further divisions to transform into primary oocytes (Figure 3.1a), which ultimately will develop into eggs. This process is known as oogenesis.

Primary oocytes continue to develop through the process of pre-vitellogenesis. This stage is marked by accumulation of cytoplasm (ooplasm), growth of the nucleus and formation of a follicle around the oocyte. KGW oocytes are about 150µm in diameter after completing pre-vitellogenesis (Figure 3.1b). A feature of this stage is the appearance of distinct vesicles referred to as cortical alveoli within the ooplasm.

Oocytes continue to develop through vitellogenesis. During this process, yolk protein accumulates as yolk globules, which fill the ooplasm and ultimately provide the nutrient and energy reserves required for embryonic development. Lipid droplets containing mainly neutral lipids (triglycerides) also begin to accumulate before the appearance of these yolk globules (West, 1990). Towards the end of oocyte development, the small lipid droplets generally coalesce to form several larger droplets which in turn, form the single oil droplet found in fully developed eggs (De Vlaming, 1983; West, 1990). In KGW, vitellogenesis is complete when oocytes attain a size of 400–450µm (Figure 3.1c). Oocytes remain this size until fish are stimulated by an appropriate environmental cue. A sharp rise in gonadotrophin initiates oocyte maturation causing the follicular envelope to release a maturation-inducing steroid, which stimulates final oocyte maturation. Final oocyte maturation is rapid in most teleosts, generally completed within 24 hours (Selman and Wallace, 1989).

In KGW, during final oocyte maturation, the germinal vesicle (nucleus) moves from its central position to the animal pole at the edge of the oocyte, next to the micropyle (opening through which the sperm enters). After this, the yolk and lipid droplets coalesce separately,

forming a single oil droplet and the ooplasm is displaced to the rim surrounding the single continuous body of yolk, resulting in increased transparency characteristic of marine finfish eggs.

The germinal vesicle (GV) membrane also breaks down during the process and the contents are released into the cytoplasm at the animal pole. At this point the delayed first meiotic division is completed. A second meiotic division begins and then is arrested until fertilisation.

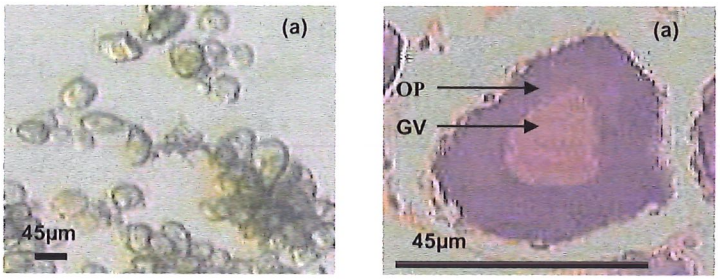
The hydration process then begins with oocytes absorbing water, increasing in size to about 800µm in diameter (Figure 3.1d). At the end of the final maturation process, the oocyte is ovulated from the follicle into the lumen of the ovary. Ovulation is achieved by contraction of the walls of the follicle. Following appropriate environmental and behavioural cues, oocytes can then spawn into the water column ready to be fertilised.

Fertilisation occurs with a single sperm crossing the chorion through the micropyle, after which the head of the sperm is incorporated into the cytoplasm (Gilkey, 1981). The chorion then hardens, and water is absorbed, forming the perivitelline space (Blaxter, 1988). This is a result of shrinkage of the egg membrane, away from the chorion, due to osmotic distension caused by discharge of colloids from cortical alveoli into the perivitelline space which occurs during the cortical reaction following fertilisation (Laale, 1980).

Macroscopic view **Microscopic view**

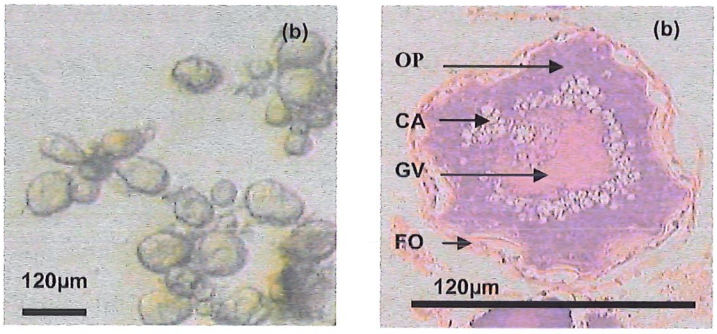
a. Primary oocyte (unyolked)

Diameter 40–60µm. Macroscopically oocytes cannot be seen without magnification. Ovaries appear glassy, grey-pink and jelly like. At this stage oocytes have a centrally located nucleus that seems large in comparison to the surrounding ooplasm.



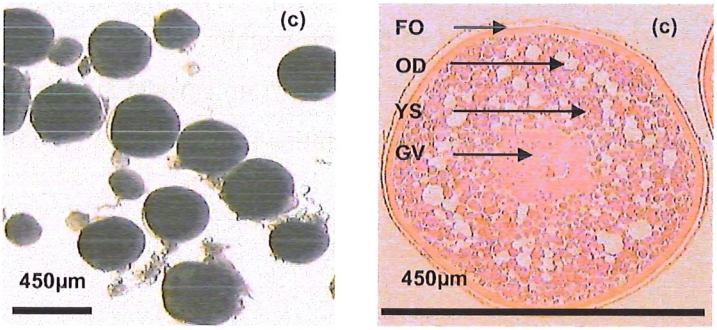
b. Pre-vitellogenic oocyte (unyolked)

Macroscopically similar to the previous stage although the size range has increased to 120–150µm. Oocytes have cortical alveoli surrounding the nucleus which will later be displaced to the periphery during vitellogenesis.



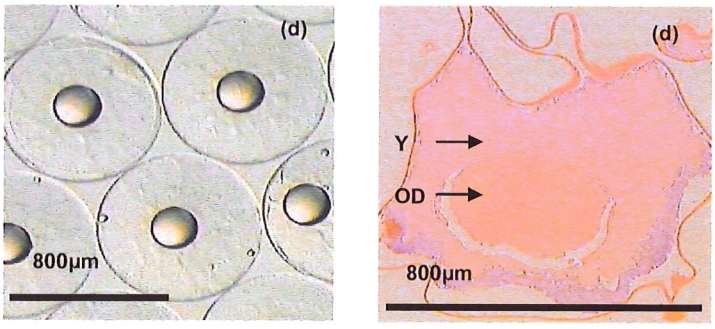
c. Vitellogenic oocyte (yolked)

Diameter 400–450µm. Oocytes visible without magnification. Oocytes are yellow in colour with small white/grey centres. Vitellogenic oocytes are marked by scattered yolk and oil spheres that will later consolidate to form a fluid yolk mass and oil droplet.



d. Hydrated oocyte

Diameter 800-850µm. Oocytes are fully hydrated and ready to be released into the water column. Yolk globules and oil droplets have coalesced to form a homogenous mass of fluid yolk containing a single oil droplet. The oocyte is transparent.



OP = Ooplasm; GV= Germinal vesicle (nucleus); CA= Cortical alveoli; FO= Follicle; OD = Oil droplet; YS= Yolk sphere; Y= fluid yolk mass

Figure 3.1 Development series of KGW oocytes from primary oocyte through to hydrated oocyte (both macroscopic and microscopic view).

3.1.2 Egg Development in KGW

Introduction

Prior to commencing larval culture of a species, it is necessary to have extensive knowledge of the biology and development of both egg and larvae. A clear understanding of egg development allows eggs to be monitored throughout incubation. If development is found to be severely abnormal, then incubation should cease and the batch of eggs discarded. Monitoring eggs in conjunction with other water quality checks (e.g. hatching success) ensures that only good quality larvae are stocked into rearing systems, reducing the likelihood of investment of effort into poor quality batches. A KGW egg development key provides a clear development sequence of fertilised KGW eggs through time.

An egg development series is also a useful tool in fisheries research. Fisheries studies estimate the spawning biomass of a fish population, to provide information for fisheries models. The Daily Egg Production Method (DEPM) is one way of estimating the spawning biomass of a fishery (Fowler, 2000). To apply this method it is necessary to determine the age of eggs from wild plankton samples, based on their stage of development (Fowler, 2000). An egg development series provides the necessary information to accurately identify KGW eggs, and determine their age, based on the stage of development, at a water temperature that is representative of those encountered by developing eggs in the wild.

Aim

To identify and document each development stage of KGW eggs from fertilisation through to hatch.

Methods

KGW eggs were collected after a natural tank-spawning. Eggs were placed into a 160L-incubation tank with low levels of water flow and aeration provided. The water temperature was maintained at 18°C, in line with temperatures experienced during spawning. 18°C is also representative of water temperatures experienced in the wild during spawning events and therefore provides results that are relevant to fisheries studies.

A sample of eggs were periodically removed from the incubator and placed under an image analysis machine. The stage of the egg was determined and photographed. The time from fertilisation to the stage of development was recorded. During incubation of KGW eggs,

photos were taken at different stages to construct a key for the development of KGW eggs from fertilisation through to hatch.

Results/discussion

A development sequence of KGW eggs from fertilisation through to hatch (Figures 3.2 – 3.21) was constructed at 18°C.

Egg development in KGW follows the same sequence found in most marine finfish that spawn pelagic eggs. This is to be expected, as only minor differences, in appearance and sequence of development, occurs in most teleost fish until hatch. These differences relate mainly to the time for gastrulation and neurulation, such that incubation may vary from one day to several weeks (Osse and Van Den Boogaart, 1995). Egg development is species-specific and water temperature is the most dependent variable parameter. Processes that occur during incubation include cleavage, formation of cell layers and morphogenesis.

The embryo develops as a blastodisc (Figure 3.10), which engulfs the surface of the yolk from the animal pole until closure of the blastopore at gastrulation and subsequent differentiation of the embryo. As hatching approaches, the embryo becomes increasingly more active and the chorion softens due to secretion of enzymes from hatching glands (Blaxter, 1988).

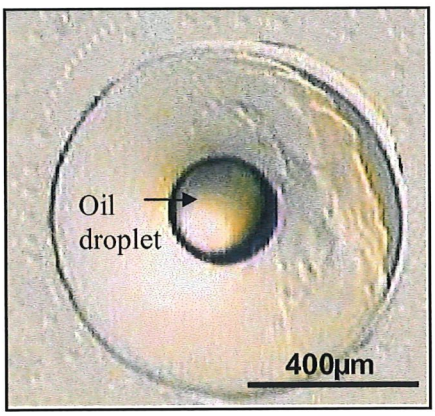


Figure 3.2 Stage 1 KGW egg.

Stage 1

Undifferentiated.

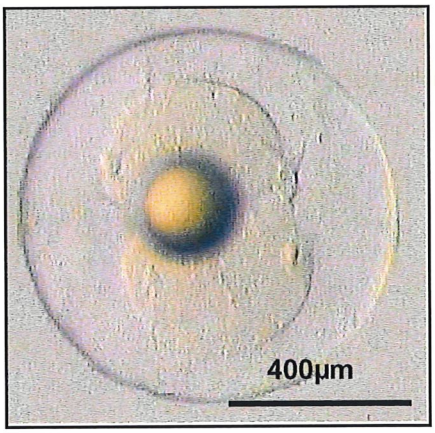


Figure 3.3 Stage 2 (2 cell) KGW egg.

Stage 2

2 cell stage, 45 minutes after fertilisation.

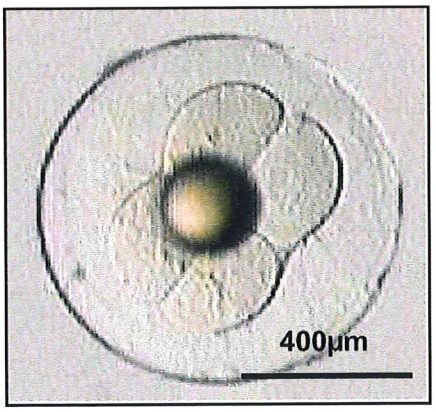
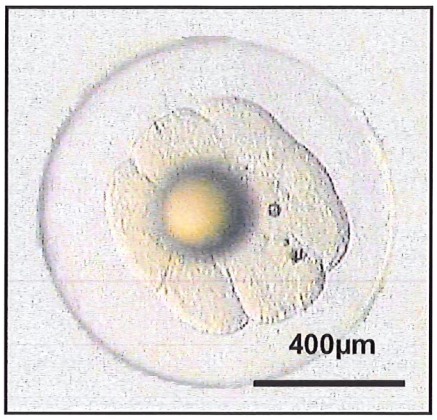


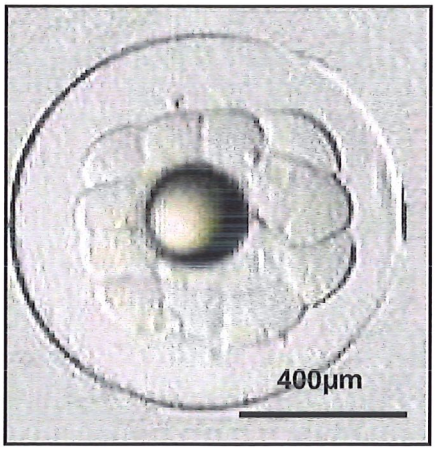
Figure 3.4 Stage 2 (4 cell) KGW egg.

4 cell stage, 1.5 hours after fertilisation.



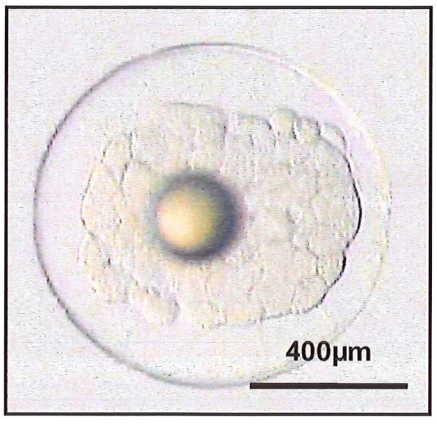
8 cell stage, 2.5 hours after fertilisation.

Figure 3.5 Stage 2 (8 cell) KGW egg.



16 cell stage, 3 hours after fertilisation.

Figure 3.6 Stage 2 (16 cell) KGW egg.



32 cell Stage, 3.5 hours after fertilisation.

Figure 3.7 Stage 2 (32 cell) KGW egg.

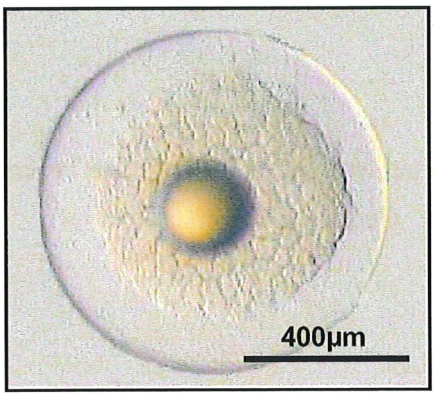


Figure 3.8 Stage 3 KGW egg.

Stage 3

4.5 hours after fertilisation. An indiscernible number of cells are evident.

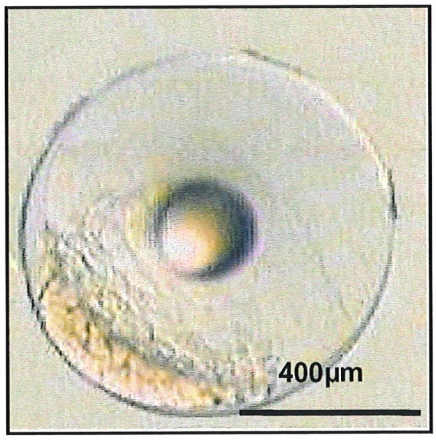


Figure 3.9 Stage 4 KGW egg.

Stage 4

7.5 hours after fertilisation. A distinct blastodisc has formed, consisting of many small cells.

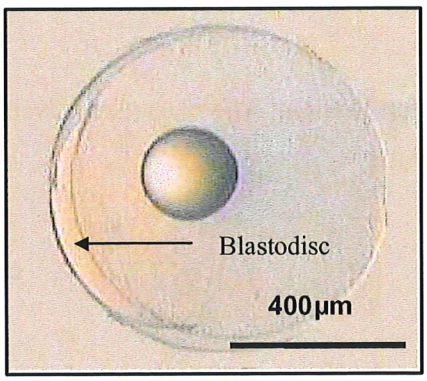


Figure 3.10 Stage 5 KGW egg.

Stage 5

12.5 hours after fertilisation. Blastodisc form a distinct cap but not yet covering 1/3 of yolk.

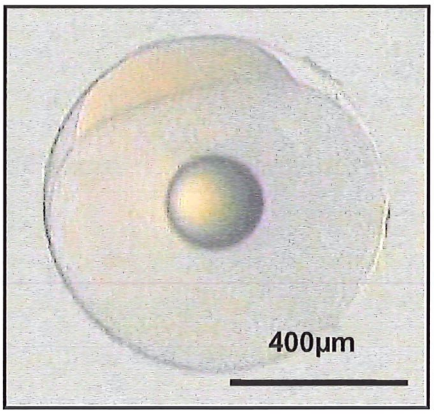


Figure 3.11 Stage 6 KGW egg.

Stage 6

14.5-15 hours after fertilisation. Blastodisc covers about 1/3 of yolk. Germ ring forming.

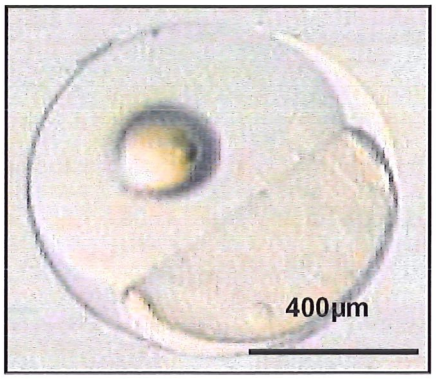


Figure 3.12 Stage 7 KGW egg.

Stage 7

18.5 hours after fertilisation. The blastodisc covers about 1/2 of the yolk.

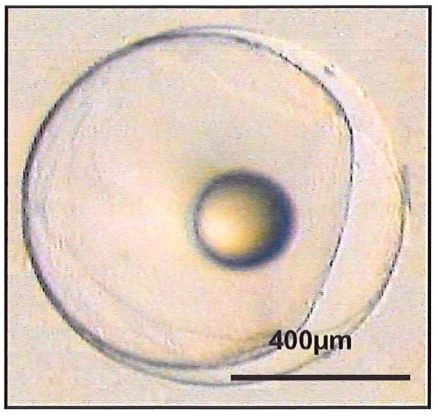


Figure 3.13 Stage 8 KGW egg.

Stage 8

21.5 hours after fertilisation. The blastodisc covers about 3/4 of the yolk.

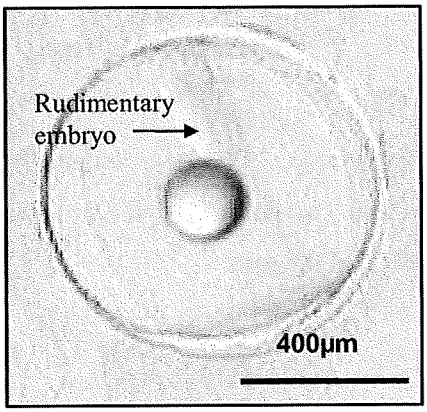


Figure 3.14 Stage 9 KGW egg.

Stage 9

24.5 hours after fertilisation. Rudimentary embryo present, not raised above the yolk surface.

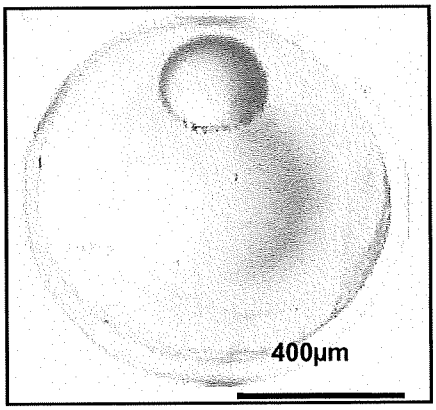


Figure 3.15 Stage 10 KGW egg.

Stage 10

28 hours after fertilisation. Embryo more developed and raised thinly above the yolk surface, optical vesicles evident, mysotomes visible, pigment spots present on both embryo and oil droplet.

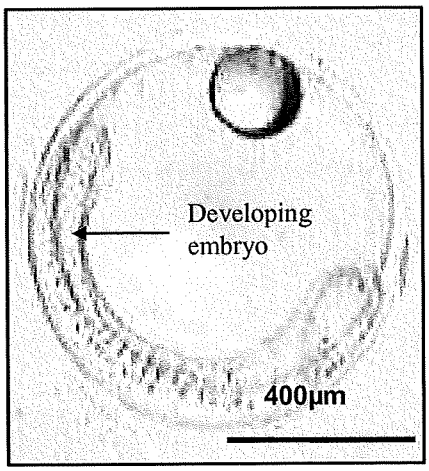


Figure 3.16 Stage 11 KGW egg.

Stage 11

35 hours after fertilisation. Embryo well developed, rounded and thick along its entire length, pigment spots very prominent, tail blunt but not yet lifted from the yolk.

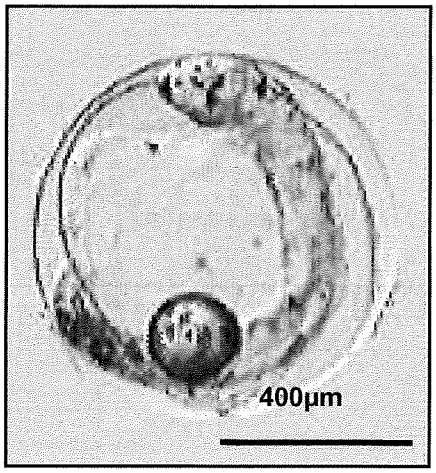


Figure 3.17 Stage 12 KGW egg.

Stage 12

41 hours after fertilisation. Embryo about 3/5 around the yolk and its entire length and tail budding starting to lift at the root.

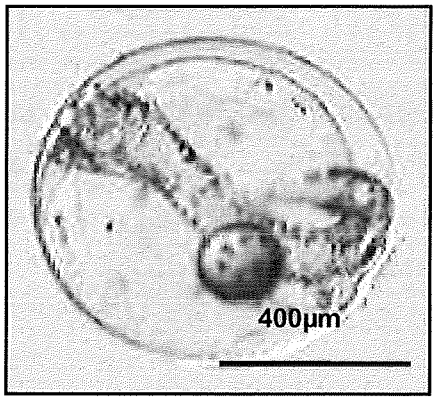


Figure 3.18 Stage 13 KGW egg.

Stage 13

43.5 hours after fertilisation. Tail lifted from yolk and starting to turn away from the mid-line of the embryo. Embryo about 3/5 around the yolk.

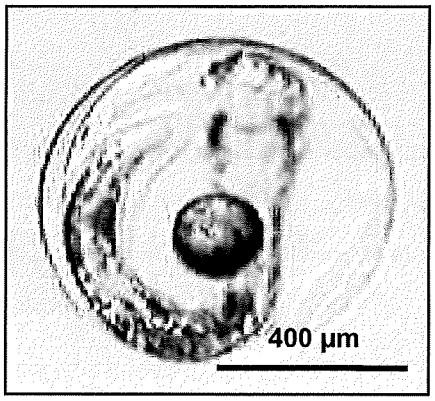


Figure 3.19 Stage 14 KGW egg.

Stage 14

48 hours after fertilisation. Tail pointed away from the mid line of body halfway along the embryo.

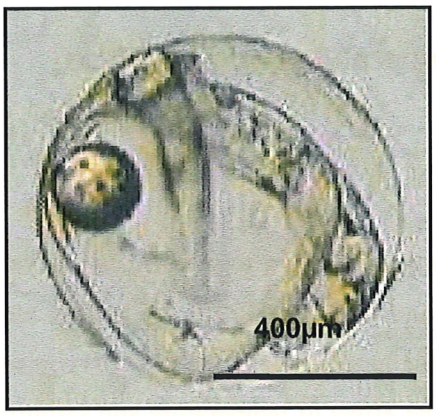


Figure 3.20 Stage 15 KGW egg.

Stage 15

52 hours after fertilisation. Embryo orientated at one pole of the yolk sac – off centre from the line of the oil droplet.

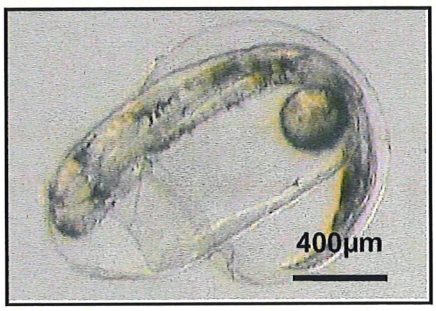


Figure 3.21 Hatching of KGW larvae.

Hatch

54 hours after fertilisation. The embryo is fully developed and breaking free from the chorion of the egg.

Eggs in this development series were incubated at 18°C. Egg development stages as per Fowler (2000).

3.2 Maximising hatch of KGW at elevated temperatures and salinities throughout incubation

Introduction

Environmental parameters such as temperature and salinity are known to affect the survival and development of eggs during hatching (Mihelakakis and Kitajima, 1994). If large scale larval rearing is to be attempted, it is important to optimise hatching conditions to maximise survival and achieve normal development. Establishing the effect of temperature and salinity on hatching success, and time to hatch, will indicate the optimum conditions for egg incubation in aquaculture and may also have implications in fisheries studies of egg and larval dispersal. The effect of temperature and salinity on the survival and development of eggs during incubation was therefore assessed.

The initial study (Trial 1) investigated the development and survival of KGW eggs at elevated temperatures and salinities at varied physiological times (i.e. time (hours) x water temperature (°C)) through the hatching process.

Experiences from trial 1 exposed a need to better maintain salinity. A second trial (Trial 2) was conducted to improve salinity stability and extend the range of temperature and salinity combinations that maximise KGW hatching success, identify the upper thermal tolerance limit of KGW eggs, and document the time to hatch at each combination.

3.2.1 Trial 1

Aim 1

To investigate the development and survival of KGW eggs at elevated temperatures and salinities at varied physiological times through the hatching process.

Aim 2

To determine the optimum temperature and salinity which maximises the hatching success of KGW eggs throughout incubation.

Methods

Experimental larval rearing system

An experimental system was constructed to investigate the hatching and early larval rearing of KGW. The system consisted of twenty 30L hemispherical tanks (larval rearing tanks) mounted in 200L water baths (Figure 3.22). The system allowed five water temperature treatments (20, 22, 24, 26, 28°C) to be investigated with four replicates of each.

Water temperature was maintained using a building automation system (BAS), that controlled the delivery of hot, cold and ambient seawater to each tank and water bath. Four replicate larval rearing tanks were randomly assigned to each of five BAS controlled water temperature outlets. Five plastic containers (700mL) were suspended in each tank, to allow five different salinities to be investigated within each larval rearing tank (Figure 3.2.2) The experimental design (3.23) was replicated 4 times.

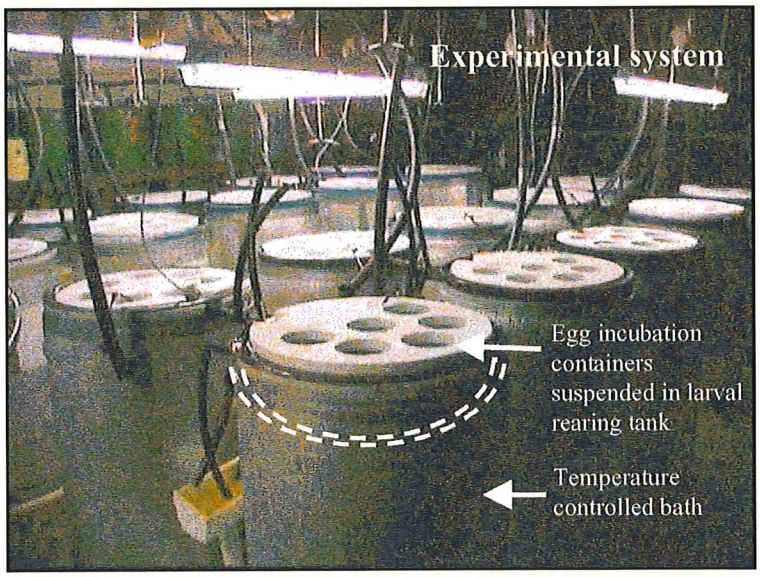


Figure 3.22 Experimental systems used to investigate the effect of water temperature and salinity on the hatching of KGW eggs.

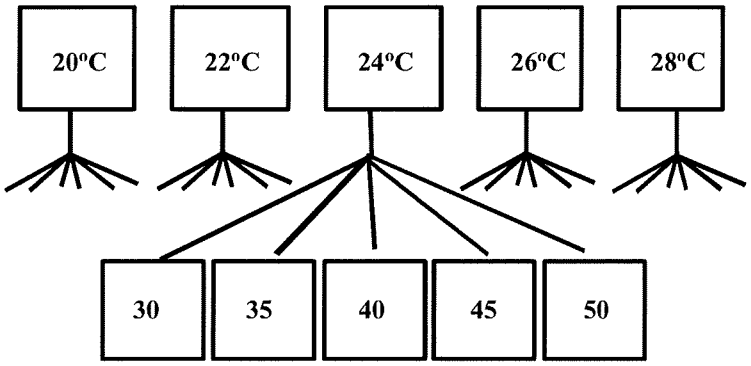


Figure 3.23 Experimental design used to investigate the effect of water temperature and salinity on the hatching of KGW eggs.

Five water temperatures (20, 22, 24, 26 and 28°C) and five salinities (30, 35, 40, 45 and 50) were established within the experimental system outlined above. The lower water temperatures investigated (20°C to 22°C) were representative of these experienced in the wild during egg development (17 - 19°C), whilst higher temperatures (25 and 28°C) were examined to investigate the upper thermal limit for KGW eggs. The salinities ranged from lower than that of seawater (30) to a level (50) that could possibly be experienced in high temperature, shallow areas of Spencer Gulf known to house larval KGW. Four replicate larval rearing tanks were randomly assigned to each of the five water temperatures. Each tank contained five x 700mL plastic containers, each filled with 500mL of water maintained at one of the salinities to be tested. All filled containers were placed into tanks 24 hours prior to stocking. The water temperature within each tank was recorded on a database every hour in addition to a manual recording once a day.

The beginning of the experiment (16/2/99)

KGW eggs were collected at approximately 6:00 am on the 16 February 1999 after a voluntary tank spawning. Broodstock had been injected with LHRHa (des-gly^{16},D-trp^{6}, pro^{9} - ethylamide) at a concentration of 10µg/kg body weight, on the 14 February 1999 and were maintained in a broodstock facility at 18°C.

Egg quality was checked and a volumetric method for counting eggs. 100 eggs were stocked into each of the containers.

Five eggs were removed from each of the containers and placed into 5 % formalin at 0250, 0400, 0800 and 1200°hrs (physiological hours – temperature times by the actual hours).

Remaining eggs were maintained until hatch. Hatch-time was considered at 50% hatch (approximately 3 hours after the onset of hatching) at which time eggs and larvae from each container were placed into 5% formalin to preserve eggs and larvae for subsequent counting.

The number of dead and developing eggs were recorded for each sampling occasion (staging was conducted as per Fowler, 2000). Eggs that had a fully developed embryo and were in the process of hatching were considered as a 'hatch' in our results. Mean survival was calculated for each temperature, salinity combination at different physiological hours.

Analysis of variance and posthoc testing at the 5% significance level was conducted by Biometrics SA (Partington, 1999).

3.2.2 Trial 2

Aim 1

To determine the optimal water temperature and salinity combination to maximise the hatching success of KGW eggs using an improved experimental protocol. Specifically, document the time to hatch at different water temperatures and the percentage hatch under different temperature and salinity combinations.

Methods

Five water temperatures (16, 19, 22, 25 and 28°C) and five salinities (30, 35, 40, 45 and 50) were established within the experimental system outlined in trial 1. The availability of cooler water during this trial allowed lowered water temperatures (16°C and 19°C) which are likely to be experienced in the wild, to be investigated. Temperatures up to 28°C were examined in this study to further evaluate the upper limit for KGW eggs during incubation. Four replicate larval rearing tanks were randomly assigned to each of the five water temperatures. Each tank contained five x 700mL plastic containers, each filled with 500mL of water at one of the salinities to be tested. All filled containers were placed into tanks 24 hours prior to stocking. Lids were placed onto each of the containers to stop evaporation and salinity fluctuations.

Water temperature within each tank was monitored manually every day and recorded automatically on a database every hour.

KGW eggs were collected after a natural tank spawning (18.5°C) at approximately 7:30pm (12 May 1999). Egg quality was checked visually and a volumetric method for counting eggs was conducted. Approximately 130 eggs were stocked into each container by transferring a measured volume of agitated eggs.

Eggs were maintained until hatch. Hatch was considered to be at 50% hatch (approximately 3 hours after hatching) at which time eggs and larvae from each container were placed into 5% formalin for subsequent counting.

The number of dead, developing and hatching eggs was recorded for each container. Eggs that were still developing were staged (staging was conducted as per Fowler, 2000). Eggs that had a fully developed embryo or were in the process of hatching were considered as a hatch in our results.

Analysis of variance and posthoc testing at the 5% significance level was conducted by Biometrics SA (Partington, 1999).

Results and discussion (trial 1 and 2)

In trial 1 salinity and seawater temperature had an interactive affect on the hatching success of KGW eggs during incubation ($P<0.0001$ as per ANOVA Table A1.1 in Appendix 1). The effect of temperature appeared to be more dominant than salinity. However, salinity varied somewhat for treatments as the containers were not covered throughout this study and evaporation occurred. The evaporation rate was not consistent throughout containers.

Hatching success was greatest at 20 and 22°C, at salinities between 35 to 45, peaking at 40 (Figure 3.24). This pattern was reflected in the mean survival of developing eggs at 0250, 0400 and 0800°hrs (Figure 3.25). The effect of salinity on the survival of KGW eggs was less evident than the effect of temperature (Figure 3.25). Natural KGW spawning in South Australia seems to occur between seawater temperatures 17°C to 19°C (Fowler *et al*, 1999). The optimum temperature range examined in trial 1 is slightly higher than the temperatures KGW eggs are likely to experienced in the wild. Additional information on the effect of lower temperatures (16°C to 20°C) should be collected, as these may be more representative of seawater temperature experienced in the wild during this stage of development.

High mortality was recorded at 26°C and total mortality was recorded for the 28°C treatment beyond 0250°hrs in trial 1 (Figure 3.25), clearly indicating that temperatures $\geq$ 26°C are not suitable for the incubation of KGW eggs regardless of the salinity. The 24°C treatment had varied results both within and between salinities (Figure 3.24). When the results of individual containers were compared with temperature data, it was apparent that replicates that had fallen below 24°C had higher survival than those replicates that had fluctuated above 24°C. This pattern suggests that 24°C may be approaching the upper thermal limit (the highest temperature at which eggs can survive) for the incubation of KGW eggs.

Temperature treatments 25°C and 28°C in trial 2 experienced almost total mortality prior to hatch, across all salinities (Figure 3.26). These results supported the findings of the initial trial (3.2.1 Trial 1) whereby large-scale mortality of developing eggs occurred at seawater temperatures >24°C.

Results from ANOVA (Table A1.2 in Appendix 1) indicate that water temperature and salinity had both an independent and interactive effect on hatching success ($P<0.001$). Hatching success was optimised in trial 2 in temperature treatments 16°C and 19°C in salinity treatments 40 to 50 (Figure 3.26). Salinities $\leq$30 caused eggs to become negatively buoyant. The trend of hatching success at 20°C and 22°C in salinities 35 to 45 was consistent with hatching success at 19°C and 22°C in salinities 35 to 45 achieved in trial 1 and 2 respectively. Results in 16°C treatments at a salinity of 35 in trial 2 seem inconsistent with results from both trials for other temperatures (Figure 3.26).

Time to hatch in actual hours was shortest in 22°C treatments and longest in 16°C treatments. A similar pattern was achieved with time to hatch in physiological hours (time (hours) x water temperature (°C)) (Figure 3.27).

Results from these trials suggest that hatching success for KGW eggs is optimised when the temperature and salinity is maintained between 16°C to 22°C and 35 to 45 throughout incubation.

The results indicate that KGW eggs can hatch at higher salinities than most marine finfish (generally marine finfish will be stressed by salinities of 40 or higher (Tucker, 1998)). The reduced hatching success in salinities below 35 is consistent with expectations of the survival of marine eggs at lowered salinities ($\leq$30). Lowered survival rates at these salinities may also

have been attributed to additional stress and disease complications that can exist when eggs become negatively buoyant.

The optimum hatching temperatures identified in this study (16-22°C) incorporates and extends beyond (-1°C, +3°C) temperatures that would be likely to be experienced during the natural spawning season in South Australia (17-19°C; Fowler, 2000). Limitations to cold water access in our systems restricted the minimum temperature investigated and, consequently, it was not possible to explore temperatures lower than 16°C. Results from 16°C temperature treatments have shown that at this temperature KGW have extended incubation periods while providing no improvement in hatching success. Temperatures lower than 16°C are likely to have their incubation periods further extended, that could increase the opportunity for diseases to develop, while occupying hatchery facilities. It is unlikely that commercial hatcheries would use lowered temperatures unless a significant improvement in larval quality could be achieved therefore temperatures below 16°C were not further investigated.

Optimum temperature and salinity ranges identified in these trials (16°C to 22°C and 35 to 45) incorporate the temperature and salinity to which the source broodstock were exposed (~18°C at a salinity of 37). This suggests that hatching success is optimised when environmental conditions (eg. temperature and salinity) are consistent with those experienced by broodstock and hence eggs at the time of spawning.

Additional information regarding the quality of larvae after hatch is desired, as the environmental conditions during incubation are known to affect larval quality (Tucker, 1998).

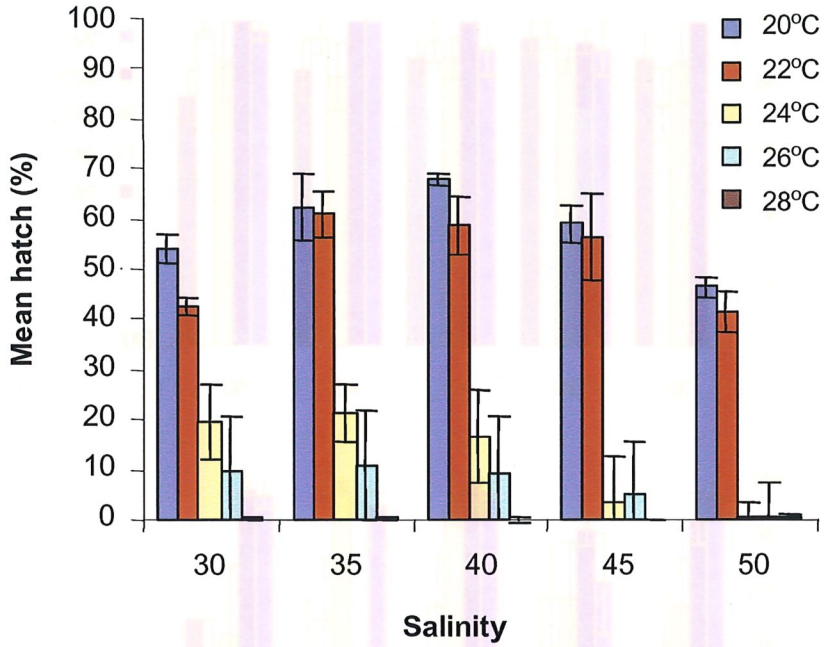


Figure 3.24 Mean hatching success (%) at different water temperature and salinity combinations (±SE) in trial 1.

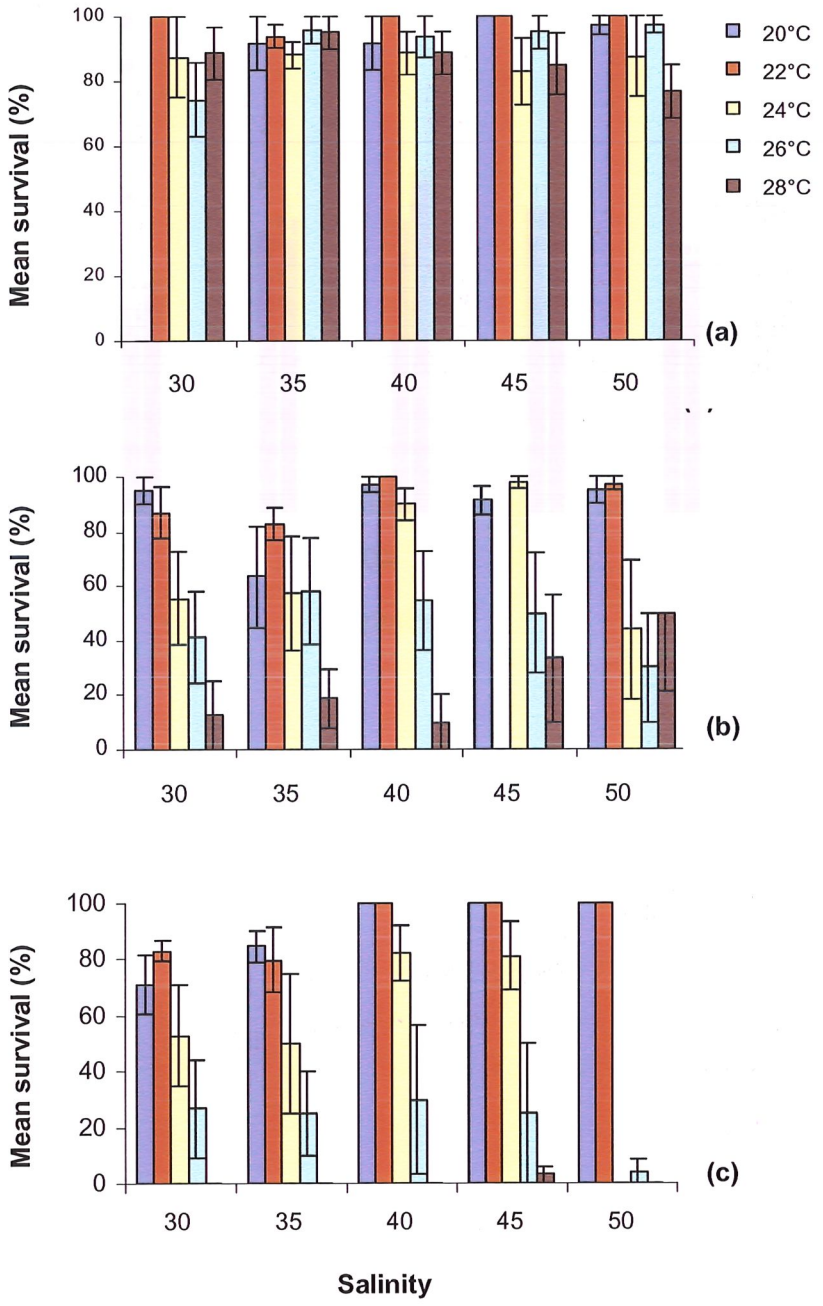


Figure 3.25 Mean survival (%) of KGW eggs at (a) 250, (b) 400 and (c) 800°hr incubated at different water temperatures and salinities (±SE) in trial 1.

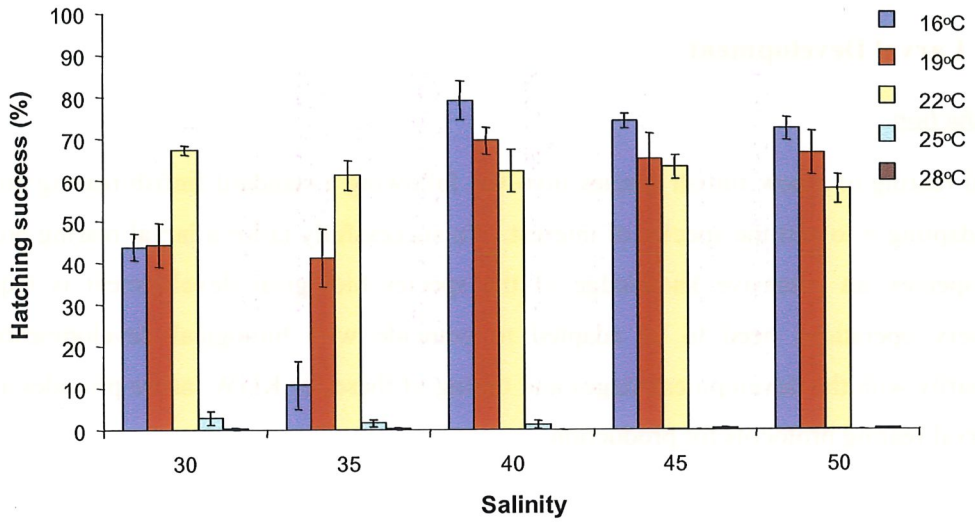


Figure 3.26 Mean hatching success (%) of KGW eggs in seawater temperatures treatments (16°C, 19°C, 22°C, 25°C and 28°C) and salinities treatments (30, 35, 40, 45 and 50) (±SE) in trial 2.

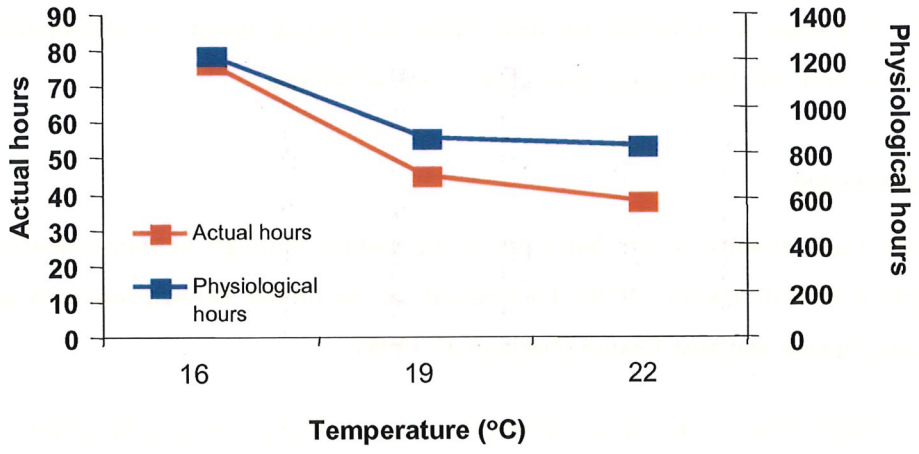


Figure 3.27 Time to hatch in 16,19 and 22°C treatments in actual hours and physiological hours (actual hours x temperature °C) for KGW eggs in trial 2.

3.3 Early larval development

3.3.1 Larval Development

Introduction

Larval rearing of a new finfish species involves following a standard finfish rearing protocol and adapting it to suit the species of interest. To successfully tailor a larval rearing protocol to a species, an extensive knowledge of the species biological development is required. Hatchery operations need to be adapted to coincide with biological developments. A familiarity with the development stages and timing of these for KGW larvae provides a basis for larval rearing protocols for production.

Methods

Photos of larvae at different developmental stages were taken during routine larval rearing runs at SARDI to construct a key on the development of KGW larvae.

KGW larvae (hatching success rate >80%) were placed in 1600L tanks at 20°C. Low levels of water flow and aeration were provided.

A sample of larvae (n=10) were removed from tanks daily and placed under an image analysis machine. The stage of the larvae was determined and photographed. A development key of KGW larvae from hatch through to post-flexion was developed.

Results/discussion

The larval stage broadly covers hatching of the embryo through to transformation of the larvae into a juvenile (Bond, 1979). This period can be further defined into yolk sac stage, Pre-flexion, flexion and post-flexion (Neria *et al,* 1998).

Like most marine teleosts, KGW larvae hatch at a small size (2–2.6mm total length) and have no functional gut, eyes or mouth. After hatching, larvae go through a series of physical and behavioural stages of development. Transformation into a juvenile is completed when a larvae takes on the characteristics of a adult, such as developed fins, rays, bones and scales, and have an improved ability to feed (Tucker, 1998). In the wild, KGW have larval period which extends for 80-146 days to the post settlement (early juvenile) phase (Fowler and Short, 1996). The larval period can be shortened in reared KGW larvae, depending on the

temperature and feeding regime during culture (about 70 days at 20–22°C temperature). A summary of major developmental events occurring over the first 12 days of KGW development is presented in Figure 3.28.

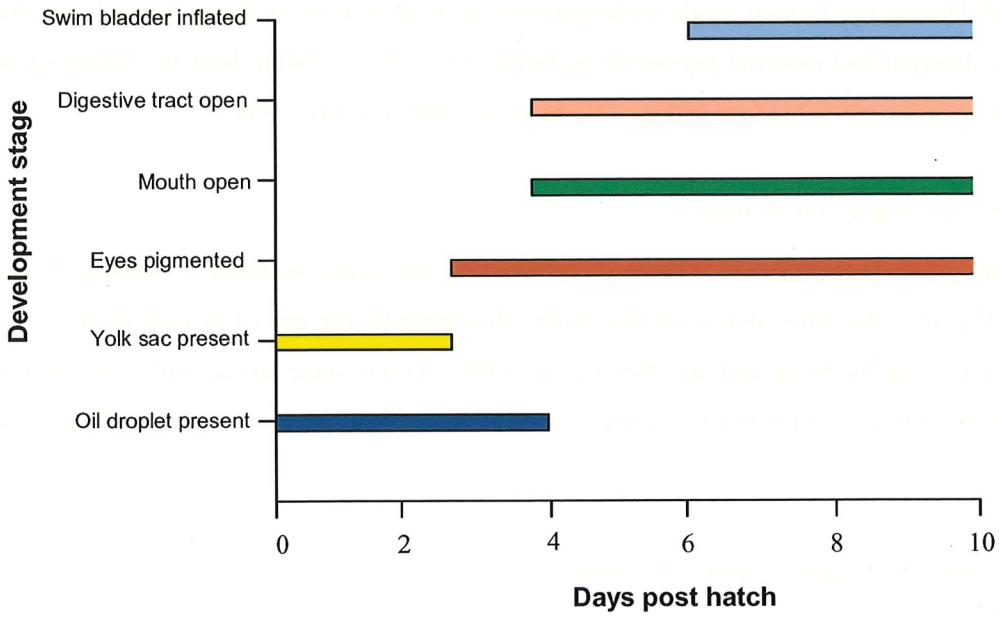


Figure 3.28 Overview of KGW early larval development stages for KGW larvae cultured at 20°C.

Yolk sac stage (1–3 days post hatch (dph); 2.4–2.9mm)

As the name suggests, this stage is characterised by the presence of the yolk sac (sac-like extension of the embryonic gut) containing the yolk, the nutritive material for embryos and newly hatched larvae, and a single oil droplet (Figures 3.29, 3.30, 3.31 and 3.32). At this stage larvae lack the ability to feed externally and all nutritional requirements are supplied from the yolk and oil droplet. Larvae in the yolk sac stage move passively near the water surface. Hatched larvae are only lightly pigmented.

Approaching pre-flexion (4 dph; 2.9-3.0mm)

As larvae approach pre-flexion, their eyes become fully pigmented and their mouths and gut (straight at this stage of development) open (Figure 3.33). The yolk sac is mostly absorbed and there is only a small remnant of oil droplet.

Pre-flexion (4–18 dph; 3.0–5.6mm)

Pre-flexion is marked by the onset of exogenous feeding as the eyes, mouth and digestive system become functional (Figure 3.34). At this stage the swim bladder begins to form (6 dph) and inflate (8–10 dph) (Figure 3.35 to 3.37). The gut continues to develop, but remains straight at this stage. The notocord is straight. Larvae begin actively swimming as they search for food. During pre-flexion single melanophores develop in a series along the mid-line trunk and tail. Internal and external pigmentation begins to develop, visible near the hindbrain and dorsally over the swim bladder and gut (Neria *et al,* 1998) (Figure 3.38).

Flexion (22–29 dph; 5.6–6.3mm)

In flexed larvae, the notochord is turning upwards and the caudal skeleton is forming (Tucker, 1998) (Figure 3.39). Most dorsal melanophores disappear by the end of flexion stage, leaving only a few along the trunk and tail (Neria *et al,* 1998). At this stage larvae start to move down through the water column but still remain in the top half of the tank, often feeding off the sides.

Post-flexion (29–50 dph; 6.3mm–14.10mm)

In post-flexion, the notochord has reached its final position and the caudal skeleton is mostly formed (Tucker, 1998) (Figure 3.40 and 3.41). Dorsal pigmentation on the trunk and tail reappears during the post-flexion stage as discrete pigment blotches, each comprising of 3-4 pairs of stellate melanophores (Neria *et al,* 1998). Post-flexion larvae begin to settle and feed in the lower half of the water column.

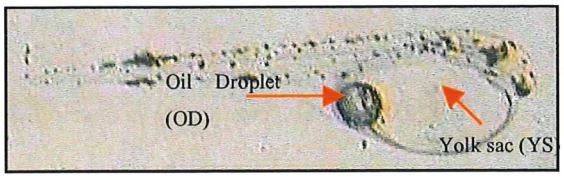


Figure 3.29 KGW larvae (0 dph).

Yolk sac stage

0 dph (hatch)

Feeding off yolk sac, oil droplet visible. TL = 2.4-2.6mm; YS = 0.9 x 0.5mm; OD = 0.2mm.

Figure 3.30 KGW larvae (1 dph).

1 dph

Feeding off yolk sac, oil droplet still visible. TL = 2.4-2.6mm; YS = 0.4 x 0.25mm; OD = 0.13mm.

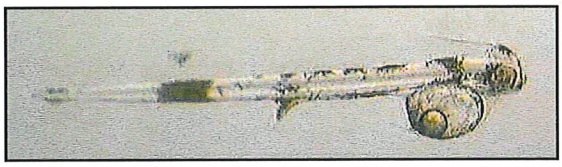


Figure 3.31 KGW larvae (2dph).

2 dph

Feeding off yolk sac, oil droplet still visible. TL = 2.6-2.8mm; YS = 0.4x0.28mm; OD = 0.14mm.

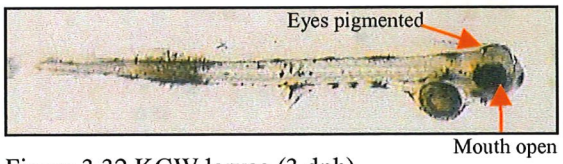


Figure 3.32 KGW larvae (3 dph).

3 dph

A small amount of yolk sac and oil droplet present. Eyes starting to pigment and mouth open.

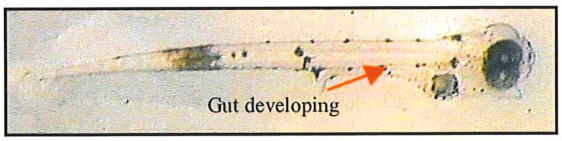


Figure 3.33 KGW larvae (4 dph).

Approaching pre-flexion

4 dph

Eyes fully pigmented, gut developing and mouth open. A small amount of yolk sac and oil droplet remains.

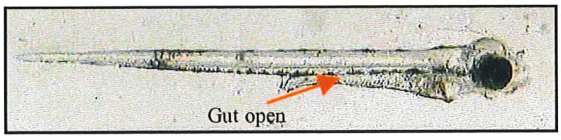


Figure 3.34 KGW larvae (6 dph).

Pre-flexion

6 dph

Oil droplet completely absorbed. Gut opening. Larvae begin to feed. TL = 3.2mm.

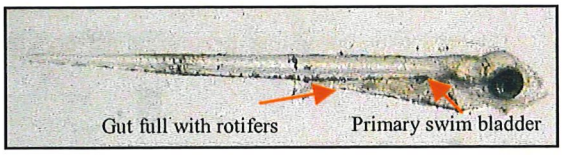


Figure 3.35 KGW larvae (7 dph).

7 dph

Gut open, full of rotifers and continuing to develop. Primary swim bladder visible. TL = 3.6mm.

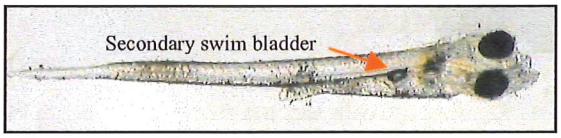


Figure 3.36 KGW larvae (8 dph).

Primary inflation

8 dph

Swim bladder visible, continuing to develop. TL = 3.4-3.6mm.

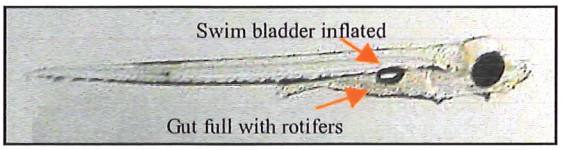


Figure 3.37 KGW larvae (10 dph).

10 dph

Swim bladder inflated. TL = 4.6.

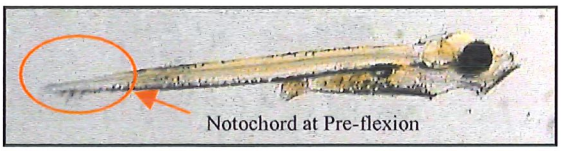


Figure 3.38 KGW larvae (18 dph).

Pre-flexion

18 dph

Increased pigmentation becomes visible. TL = 5.3mm

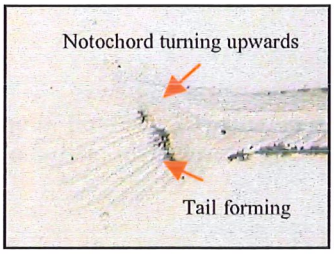


Figure 3.39 Tail of KGW larvae during notocord flexion (22dph).

Flexion

22 dph

The notochord is beginning to turn upwards as the caudal skeleton is forming. TL = 5.8mm.

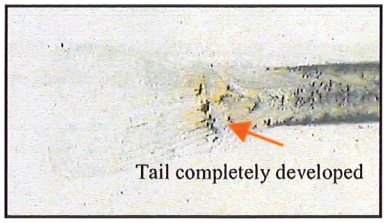


Figure 3.40 Tail of KGW larvae after notocord flexion (36 dph).

Post-flexion

36 dph

The notochord has reached is final position. Pigmentation continues and is particularly evident on the truck and tail.

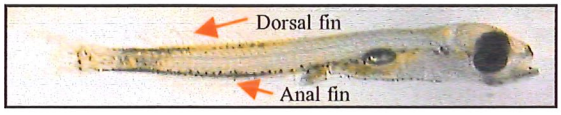


Figure 3.41 KGW larvae (36 dph).

36 dph

At this stage the caudal skeleton is mostly formed. The dorsal and anal fins are developed. Pigmentation continues becoming more evident. TL= 8mm.

YS = Yolk sac; OD = Oil droplet; TL = Total length

Larvae in this series were cultured at 20°C.

3.2.2 Examination of mouth parts of KGW larvae using clearing and staining techniques

Introduction

Larval rearing of KGW was conducted at SARDI Aquatic Sciences between 1997 and 2001. Initial trials indicated that one of the problems associated with KGW production is the poor survival of KGW larvae at first-feeding, once the endogenous feed reserves of larvae have been absorbed. At this stage, reared larvae initially commence feeding on an small live feed, rotifers (*Brachionus rotundiformis* (small strain) and *Brachionus plicatilis* (large strain)). They are then weaned onto *Artemia* and commercial dry feed products (e.g. crumble and pellets). To improve survival throughout this period, information is required about the optimum-size feeds larvae are able to ingest at different stages, particularly at first-feeding and the transition from rotifers to *Artemia*.

To determine the optimal-size feed for larvae at varied stages of development, data on jaw length is required. Studies by Shirota (1970) suggest that finfish are able to ingest prey items half the size of mouth gape where mouth gape is calculated from jaw lengths (Figure 3.42): Mouth gape = $\sqrt{}$ [(upper jaw length) ^{2}+(lower jaw length) ^{2}].

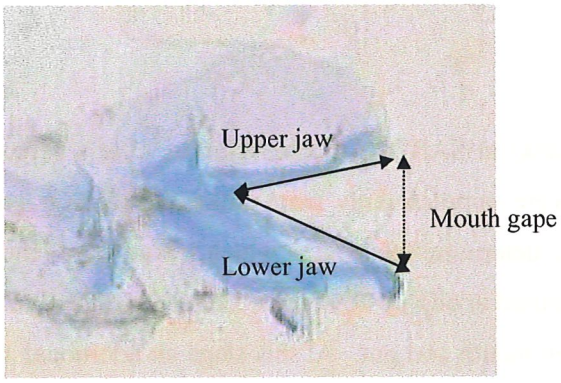


Figure 3.42 Diagram showing calculation of mouth gape of finfish larvae (6dph).

The above diagram was modified from Shirota (1970).

Jaw lengths can be easily measured in young (6-15dph) transparent larvae under a microscope. However, as larvae grow and develop, it is hard to distinguish bone from the cartilage and flesh, making it difficult to obtain accurate jaw measurements.

Accurate jaw measurements can be obtained by clearing flesh and staining to differentiate between bone and cartilage as described in Potthoff (1984). Such methods can be employed to gain more detailed insight into the skeletal structure of 'normal' fish and those displaying various types of deformities that may impede ingestion. To date, no studies have used clearing and staining techniques to examine the jaw measurements of KGW larvae and the skeletal structure of KGW juveniles.

Aim 1 - Mouth gape

To determine the mouth gape of KGW larvae at first feeding to establish the appropriate size of feed at this stage.

Aim 2 - Standard length-mouth gape relationship

To investigate the relationship between mouth gape and standard length to allow feed size to be adjusted with growth.

Aim 3 - Skeletal structure

To investigate the skeletal structure of a 'normal' KGW juvenile.

Methods

Mouth gape

KGW larvae were cultured at 19°C in 33L tanks at SARDI Aquatic Sciences after a natural tank spawning on 1 May 2000. 10 larvae were sampled daily from tanks and photographs were taken using an image-analysis system to determine the time at which larvae were ready to commence feeding. First-feeding was identified at 6dph when larvae had utilised their yolk sac, had pigmented eyes and had both an open mouth and gut. At this stage an additional 10 larvae from a total of four tanks were sampled for clearing and staining purposes. Each larva was photographed, standard length recorded (µm) and stored in a separate vial containing 10% formalin in seawater for future staining. The clearing and staining procedure (Table A1.3 in Appendix 1) was modified from Potthoff (1984).

Once staining was completed, the upper and lower jaw lengths were calculated (µm). In many cases the eye of the larvae were removed to obtain a clear view of the mouth joints. Mouth gape was calculated from jaw lengths using the formula described Shirota (1970).

Standard length-mouth gape relationship

KGW larvae were cultured at 20°C in 1600L tanks at SARDI Aquatic Sciences. 10 larvae were removed from tanks at 6, 34, 39, 49, 51 and 57 dph. Photographic images were taken of larvae and their standard length (µm) was recorded. Larvae were stored in individual vials containing 10% formalin in seawater for future clearing and staining. The upper and lower jaw lengths (µm) of each larva were recorded and mouth gape was calculated (Figure 3.42). A standard length (µm) - mouth gape (µm) relationship was determined by plotting data in Microsoft Excel™ and fitting a trend line.

Skeletal structure

100 dph juveniles (91mm to 106mm standard length) cultured at SARDI Aquatic Sciences were euthanased using an overdose of an anaesthetic (benzocaine). Scales and flesh were removed from one side of the juveniles using a scalpel. The juvenile was stored in 10% formalin for a period of 1 month. Staining of the juvenile was conducted as per Table A1.4 in Appendix 1.

Results and discussion

Mouth gape at first feeding

KGW larvae were successfully stained blue (Figure 3.42), highlighting the cartilage mouthparts. Mouth-gape size of first-feeding larvae (6dph) ranged from 487.13µm to 673.47µm in larvae measured. The mean mouth gape was 571.82µm ± 12.51 (n =20). Based on studies by Shirota (1970), larvae at this stage, are able to ingest prey items ≤ 285.91µm. This indicates that *Brachionus plicatilis* (L-type) rotifer with a mean size of 239µm (Lavens and Sorgeloos, 1996) should be a suitable prey size for first-feeding larvae to ingest (Figure 3.43). Larvae at this stage are not yet efficient predators and may experience problems capturing prey. It is therefore recommended that first feeding larvae be fed on a smaller food source (s-type rotifers - 160µm), than L-type rotifers (239µm), for a period of approximately 1 week. Once larvae are actively feeding they can begin to be fed L-type rotifers.

Future trials are needed to improve the nutritional profile of rotifers through different types and levels of enrichment and to improve growth and survival of KGW larvae.

Relationship between standard length and mouth gape

The relationship ($y= 0.2378e^{0.0571x}$; $R^2=0.81$) between standard length (µm) and mouth gape (µm) (Figure 3.43) can be used as a guide to the size of feed able to be ingested by larvae as they grow. However, research has indicated that the size of prey larvae are able to ingest is not always half their mouth gape. Results from trial 3.3.1 (Assessing the transition of rotifer feeding to *Artemia* feeding in larval KGW) show that KGW larvae with a mean standard length of 7.73mm ± 0.17 are able to ingest prey items with a mean width of 450µm ± 50 (predicted particle size able to be ingested is 200µm ± 26 based on Studies by Shirota (1970)). These results indicate that as larvae develop and refine their prey catching techniques they are able to ingest prey items greater than half their mouth gape.

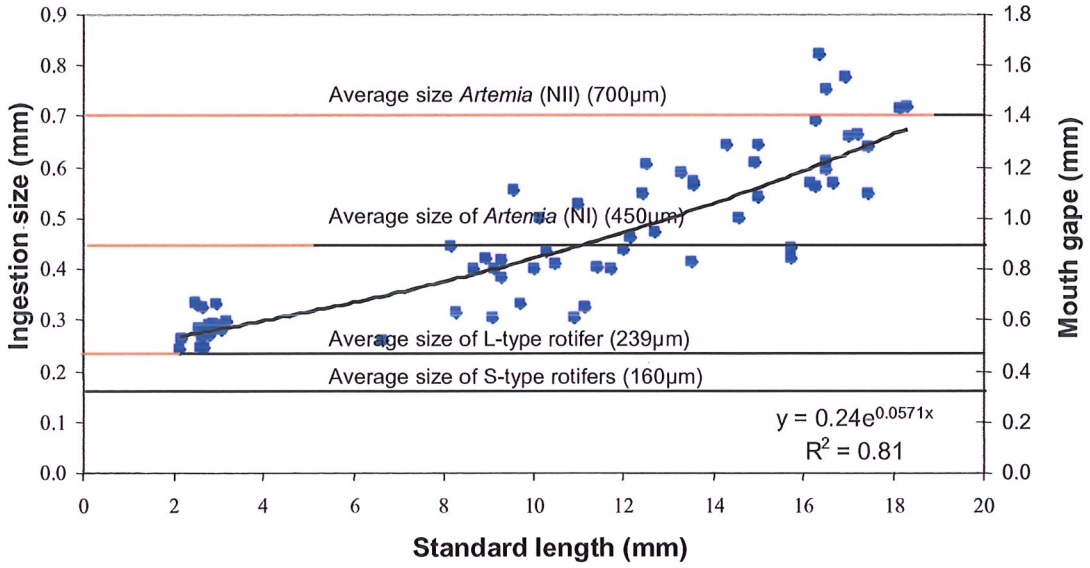


Figure 3.43 Relationship between standard length (mm) and ingestion size (mm) (calculated as half mouth gape) of KGW larvae.

Skeletal structure

The juvenile KGW were successfully stained in a combination of red and blue as shown in Figure 3.44. The red represents bone and the blue represents cartilage. The stained fish had 13 spines on the anterior dorsal fin, 1 spine and 26 soft rays on the posterior dorsal fin and 23 soft rays on the anal fin. Numerous dorsal ribs extended approximately a third of the way along the body.

These results are in line with Yearsley *et al* (1999) indicating that the skeletal structure of KGW juveniles reared in captivity is consistent with wild caught fish.

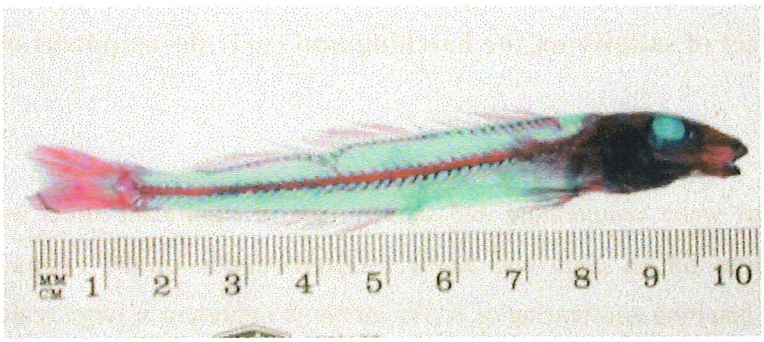


Figure 3.44 Stained KGW juvenile. Regions stained red represent bone and regions stained blue represent cartilage.

3.2.3 Investigating the effect of salinity on the hatching and early development of KGW eggs and larvae

Introduction

Environmental factors such as water temperature and salinity have a significant effect on survival and growth of marine finfish eggs and larvae. Determination of the optimal environmental conditions for hatching and rearing of KGW larvae is important if large scale rearing is to be improved.

Previous studies conducted on KGW larvae examined the combined effect of temperature and salinity on the hatching success of KGW eggs. The results from this study indicate that temperature and salinity had an interactive effect on the hatching success of KGW eggs. Results suggested that incubation temperatures between 16-19°C and salinities between 40-50 maximised hatching success.

Development of larvae to first feeding and the subsequent survival of larvae was considered in this study. Salinity was examined in isolation and temperature was maintained at 19°C (previously identified within the optimum temperature range for incubation of KGW eggs).

Aim

To examine the effect of salinity on the hatching success, yolk sac absorption larval size, mouth gape at first feeding, and the period of survival of KGW larvae during starvation.

Methods

Experimental set up

Three replicate 25L hemispherical fibreglass tanks were randomly assigned to each of the six salinities (30, 35, 40, 45, 50 and 55). Each tank was mounted onto a 200L water bath supplied with flow-through seawater set at 19°C using a building automation system to control water temperature. Seawater was manually mixed to the desired salinities using a combination of seawater, distilled water and artificial sea salt. To increase salinity by 1, 1.3g of Instant Ocean was added per litre of seawater or distilled water. 48 hours prior to stocking eggs, seawater, at the pre-established salinities, was poured into tanks. The latter were

covered with plastic sheets to reduce evaporation and thus salinity fluctuations. All tanks were gently aerated and supplied with 24-hour illumination.

Beginning of the experiment

KGW eggs were collected via a tank overflow after a natural tank spawning. Unfertilised (sunken) eggs were removed using an egg separator. A volumetric method was used to stock approximately 1000 fertile eggs into each tank. Eggs were maintained until hatch and 3 hours after the onset of hatching, 10 larvae from each tank were removed and sampled, recording total length and yolk sac dimensions. Unhatched eggs were removed, counted and the number of hatched larvae recorded. Following hatching, 10 larvae were removed daily from each daily until no larvae remained in the tanks. Total length, yolk sac dimensions (width and length) and developmental stages were recorded. Temperature, salinity and mortalities were also recorded daily. First feeding larvae (4dph) (10 per tank) were placed in 10% formalin for subsequent clearing and staining. Staining was conducted as per Table A1.3 in Appendix 1.

Once staining was completed, the upper and lower jaw lengths (µm) were calculated using an image analysis system. Where necessary, the eye of the larvae was removed to gain a clear image on the mouth joints. Mouth gape was calculated from jaw lengths using the formula described in Shirota (1970): Mouth gape = $\sqrt{}$ [(upper jaw length) ^{2}+(lower jaw length) ^{2}] [1].

Analysis of variance at 5% significance level was used to test the difference in hatching success, yolk sac volume (µm^3) at hatch, 1, 2, 3 and 4 dph at different salinities, total length (µm) at hatch, 1, 2, 3, 4 and 6dph at different salinities and mouth gape in first feeding larvae (4dph) in salinities 30 and 35. Data was observed to be normally distributed using probability plot of residuals. All data was compiled in Microsoft Excel™ and statistical analysis was performed in Statistix and GenStat.

Results/discussion

Poor survivorship (<25% across all treatments at 1dph and between 5-10% survival across all treatments by 5dph) (Figure 3.45) due to poor quality larvae, was the main factor that influenced and limited the results in this study.

Due to low survival of larvae to first feeding across all treatments (Figure 3.45), mouth gape size could only be compared between salinity treatments 30 and 35 (582.35 and 558.94µm respectively) (Figure 3.46). There was little difference between the two treatments

($P_{(2)(18)}$=0.366 as per Table A1.5 in Appendix 1) therefore no connections between rearing salinity and mouth gape and consequently increased ability to feed, could be made.

No significant difference ($P_{(5,12)}$=0.453) in hatching success was detected for the salinities tested (Figure 3.47) (ANOVA , Table A1.6 in Appendix 1). However, the trend in our results however, suggests that hatching success was highest (69 to 74.4%) in salinities between 30 to 40 and declined as salinities approached 55 (41.4% hatching success) (Figure 3.47). Some eggs in salinity treatment 55 broke apart and disintegrated before hatching. This probably occurred as a result of osmotic pressure, indicating that a salinity of 55 is approaching the upper saline limit for KGW (the highest salinity at which KGW can survive). These results are consistent with previous trials (3.2 Maximising hatch of KGW at elevated temperatures and salinities throughout incubation) where salinities between 35 to 45 and temperatures between 16°C to 19°C were identified as optimum for hatching KGW eggs. This trial had a marginally better performance in the 30 salinity treatment than in previous trials. An increase in hatching success at low salinities ($\leq$ 30), in this trial, may be partly attributed to the containers being stirred twice a day, possibly reducing the affects of metabolites and bacteria that can build up on negatively buoyant eggs.

No significant difference ($P_{(5,11)}$=0.280, $P_{(5,11)}$=0.141, $P_{(5,11)}$=0.079, $P_{(5,5)}$=0.585 and $P_{(2)(35)}$=0.999, Tables A1.7 to A1.11 in Appendix 1) was observed for the utilisation of yolk sac between treatments on any of the days tested (hatch, 1, 2, 3 and 4dph respectively. These results do not indicate any difference in yolk sac volume of KGW larvae between salinity treatments (30-55) on the days tested (hatch-4dph) (Figure 3.48).

Analysis of total length (mm) of hatched larvae indicated that there was no significant difference ($P_{(5,11)}$=0.418, $P_{(5,11)}$=0.114, $P_{(5,11)}$=0.694, $P_{(5,5)}$=0.473, $P_{(2)(35)}$=0.418 and $P_{(2)(11)}$=0.693, Tables A1.12 to A1.17 in Appendix 1) between salinity treatments on the days tested (hatch, 1, 2, 3, 4 and 6dph respectively) (Figure 3.49). These results may partly be attributed to variations within treatments due to sub-optimal larvae.

These results suggest that salinities between 30-55 did not affect hatching success, yolk sac utilisation (measured by yolk sac volume) and early larval growth (TL (mm)) of KGW larvae.

Further trials with high quality larvae need to be conducted to investigate the optimum hatching and rearing conditions for KGW eggs and larvae.

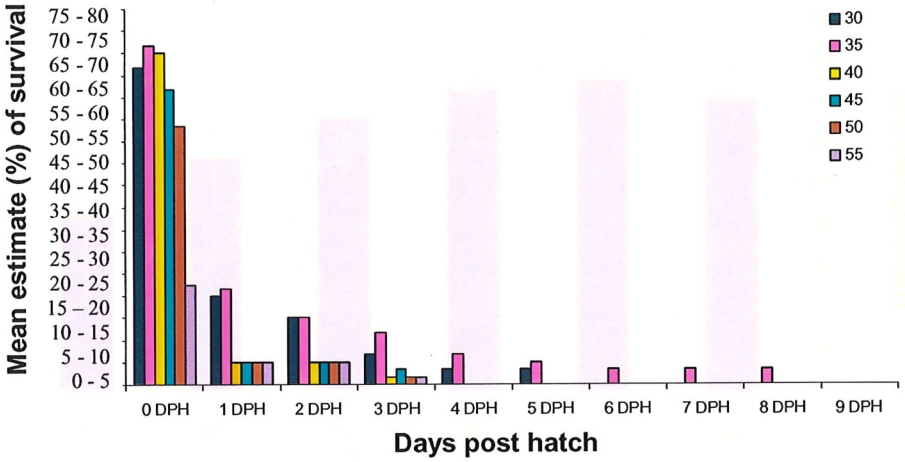


Figure 3.45 Estimated mean survival (%) of KGW larvae in different salinity treatments (30 and 35) throughout the duration (8 dph) of the trial.

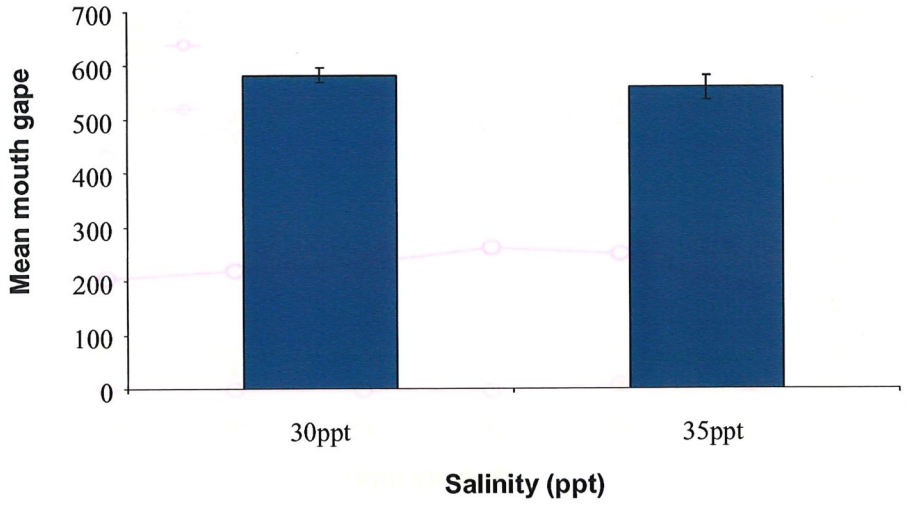


Figure 3.46 Mean mouth gape (µm) of first feeding KGW larvae in salinity treatments 30 and 35 (± SE).

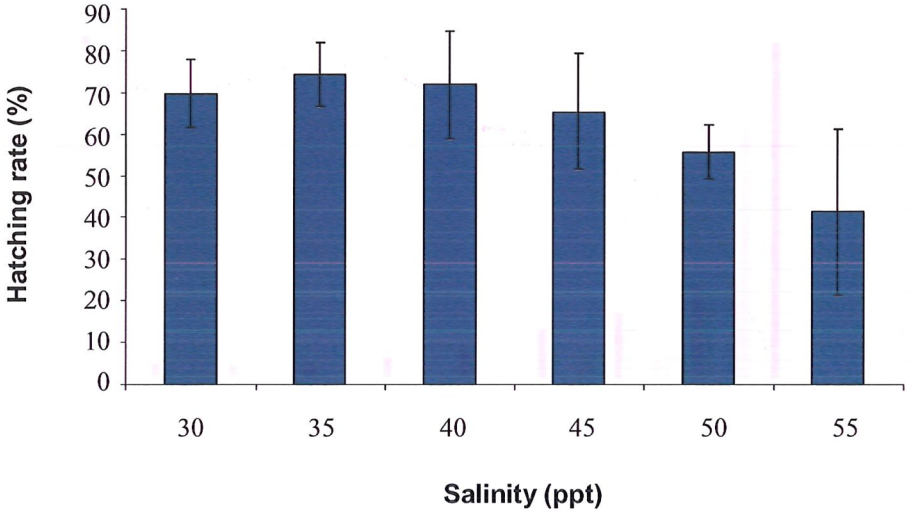


Figure 3.47 Mean hatching success (±SE) of KGW eggs incubated in different salinity treatments (30-55).

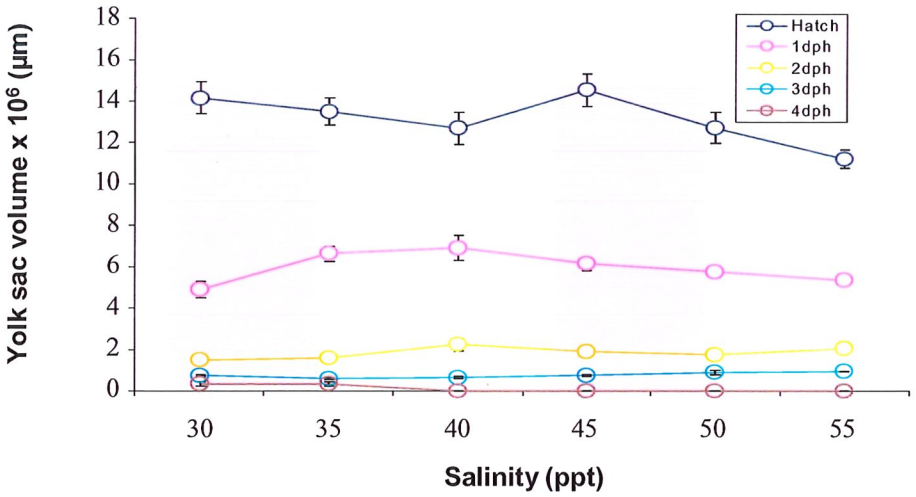


Figure 3.48 Mean yolk sac volumes (±SE) of KGW larvae in salinity treatments (30-55) over a period of 5 days.

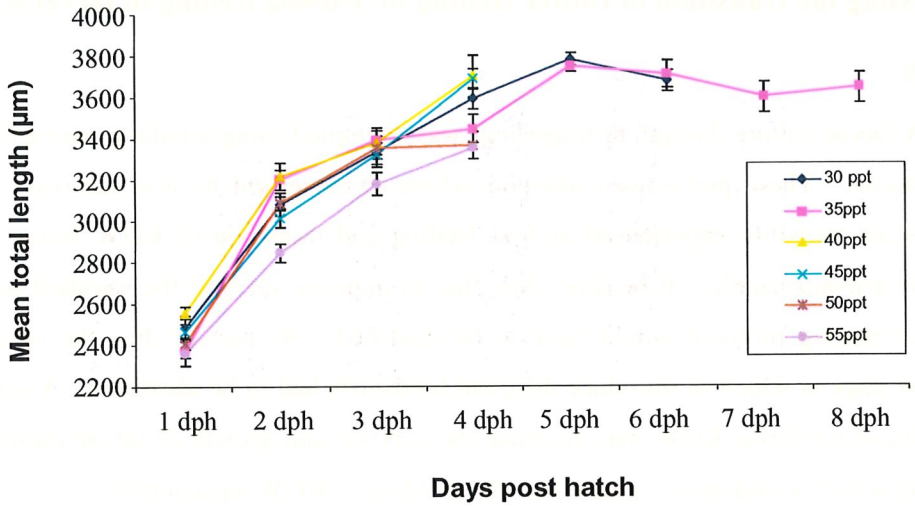


Figure 3.49 Mean total length (µm) of KGW in salinity treatments (30-55) over the duration of the trial (±SE).

3.3 Early larval feeding

3.3.1 Assessing the transition of rotifer feeding to *Artemia* feeding in larval KGW

Introduction

Initial KGW larvae culture through to fingerlings was attempted using standard marine finfish rearing protocols. These preliminary attempts where characterised by low survival (<1%) with large-scale mortality experienced at first feeding and transition of KGW larvae from rotifers onto *Artemia* nauplii. It became clear that to improve survival the standard marine finfish larval rearing protocol would have to be modified. As part of this, the optimum development stage at which to introduce different foodstuffs had to be identified. A suitable feeding protocol for larval KGW may increase the survival and growth of larvae during the larval period, which would assist in the commercialisation of KGW aquaculture.

The transition of larvae feeding on rotifers to *Artemia* is critical with respect to larval development and survival. Typically, a number of larvae die when major dietary changes such as this are made (Tucker, 1998). The timing to change the feed type must coordinate with larvae that are developed enough to locate, catch and ingest *Artemia* as well as digest and utilise *Artemia.* The ability to efficiently utilise *Artemia* is often related to the levels of digestive enzymes, which differ between individuals and with fish age and species (Tucker, 1998).

This trial is aimed at investigating the stage and size that KGW larvae could both ingest and utilise *Artemia* nauplii.

Aim

To identify the stage at which KGW larvae are able to ingest *Artemia* with respect to mouth gape and digest and utilise *Artemia* to optimise growth and survival.

Method

KGW larvae at 37 days post hatch (dph) (SL = 7.3 ± 0.29mm) were stocked at a density of 100/tank into 14 black 30L hemispherical tanks. Tanks had been seeded with micro algae, *Isochrysis galbana* (T.Iso) at approximately 400,000 cells/mL and rotifers at 10/mL to

provide a 'brown water' culture system. Water temperature was maintained at 19°C with a 16 light: 8 dark light cycle. Gentle aeration was provided to each tank. Rotifer to *Artemia* transition treatments (Table 3.1) were commenced at one week intervals with larvae in each treatment being allowed to feed on newly hatched *Artemia* (NI) for one week before progressing onto 24 hour post hatch *Artemia* (NII) enriched with DHA Selco® (INVE Aquaculture), at which point rotifer co-feeding was discontinued. *Artemia* (NI) co-feeding was continued for a further week until larvae were fed on enriched *Artemia* (NII) only. Control treatments were maintained on rotifers at 10/mL in the 'brown water' culture system for the duration of the trial. The feeding protocol for treatments 1 - 4 was conducted as per table 3.1.

Due to limitations on the number of larvae available, three replicate tanks were randomly allocated to each of the 4 treatments with 2 tanks used as controls. On the day after stocking (day 1) mortalities were removed and all tanks were re-stocked to a density of 100/tank.

During rotifer feeding the 'brown water' culture system was maintained, with micro algae and rotifers added to tanks each morning. Tanks feeding on *Artemia* (NI and NII) were maintained on a static system during the day and a slow flow-through (20% tank volume exchanged per hour (100mL/min/tank)) was used at night to flush uneaten nauplii through a 500µm screen.

Table 3.1 Schedule of live feed additions followed for treatments 1 (NI *Artemia* fed at 37dph), 2 (NI *Artemia* fed at 44dph), 3 (NI *Artemia* fed at 51dph), 4 (NI *Artemia* fed at 58dph) and control.

Treatment	Start day no. post hatch	Mean standard length (mm) ±SE	Artemia (NI) (1/mL) rotifers (10/mL)	Artemia (NI) (0.5/mL) Enriched Artemia (NII)(0.5/mL	Enriched Artemia (NII) (1/mL)
1	37	7.36 ± 0.33	Day 37	Day 44	Day 51
2	44	8.80 ± 0.40	Day 44	Day 51	Day 58
3	51	10.17 ± 0.75	Day 51	Day 58	Day 65
4	58	10.61 ± 0.65	Day 58	Day 65	Day 72
Control	Fed rotifers only throughout the trial.				

Larvae (n = 10) were sampled from each treatment tank, 24 hours after they had been offered *Artemia* (NI). Total length, standard length, upper and lower jaw measurements and gut contents (rotifers/*Artemia*) were recorded. All samples were then stored in 5% formalin in seawater for future clearing and staining investigations on jaw development. Control tank

samples were taken as each treatment commenced *Artemia* (NI) feeding, recording the measurements mentioned above. Standard length (mm) was recorded for all larvae from all tanks on the completion of the trial after 34 days (72 dph) and final larval counts from each tank were used to determine survival (%).

To identify periods of high mortality, a 20mm diameter x 20mm deep sump and a 100mm diameter white circle at the base of each tank was siphoned each morning and transferred to a 25mL conical-bottom container from which dead larvae were counted after settlement for 20-30 minutes.

ANOVA at 5% significance level was used to test the difference in percentage of larvae feeding on *Artemia* (NI), total survival and final standard lengths (mm). Tukey-Kramer HSD comparison was performed at 5% significance level to test for the source of differences in survival (%) and standard length (mm). Data was observed to be normally distributed using probability plots of residuals. Data was compiled in Microsoft Excel™ and statistical analysis was performed in Statistix7 and GenStat.

Maximum mouth gape was calculated based on the method used by Shirota (1970).

Results/discussion

No difference ($P_{(3,8)}$=0.3132) was detected from ANOVA (Table A1.18 in Appendix 1) in the percentage of larvae ingesting *Artemia* (NI) 24 hours after they were added to tanks in treatments 1 to 4 (Figure 3.50). These results indicate that KGW larvae are able to ingest *Artemia* (NI) from 37 dph (standard length (SL) (mm) = 7.36 ± 0.33 (SE)) to 58 dph (SL (mm) = 10.61 ± 0.65 (SE)). Treatment 3 had the lowest percentage of larvae feeding on *Artemia*. This result was inconsistent with treatments 1, 2 and 4.

The predicted particle size able to be ingested by a larvae with SL = 7.36mm is 0.4 ± 0.04mm (Table 3.2) (based on 50% of its mouth gape (Shirota, 1970)). The ability of larvae with a SL = 7.36mm to ingest NI *Artemia* (0.45mm) in this trial suggests that KGW larvae at this stage have become efficient predators and are able to ingest particles greater than half of their mouth gape.

The mean survival of KGW larvae (%) was affected by treatments introducing *Artemia* at approximately one week intervals ($P_{(4,9)}$=0.0017, Table A1.19 in Appendix 1). Treatments 1 and 2 that introduced *Artemia* at 37 and 44dph respectively had the lowest survival (Figure

3.51) (Table A1.22 in Appendix 1). The highest survival was experienced in treatments 3 and 4 whereby the addition of *Artemia* was delayed until 51 and 58dph respectively (Table 3.1).

Daily mortality results indicate that mortalities experienced in treatments 1 and 2 were consistently higher throughout the trial than other treatments, particularly after each change in the feeding regime (Figure 3.52). This suggests that the early introduction of *Artemia* in treatments 1 and 2 were associated with increased mortality throughout the trial. Treatment 3 experienced an increase in mortality when the feeding regime changed to *Artemia* (NII) only (65dph). However, a very low level of mortalities (<2 mean mortalities per day) occurred in treatment 4 and the control (with the exception of an unexplained spike in the control group at 66dph) throughout the trial. This suggests that the high survival rate in treatment 4 and the control could be attributed to prolonged feeding on rotifers.

Standard length (mm) was lower on completion of the trial in the control treatment (Figure 3.53) than all the treatments (1-4) fed *Artemia* ($P_{(4,9)}$=0.002) (Table A1.21 and A1.22 in Appendix 1). The increased growth in the *Artemia* fed treatments (1 – 4) is most likely associated with the improved nutrition and increased nutrient supply that can be provided through feeding both NI *Artemia* and NII *Artemia* enriched with commercial enrichment product, DHA Selco® (INVE Aquaculture). These results demonstrate the need to identify the earliest opportunity at which to transfer KGW larvae from rotifers to *Artemia* (NI) while retaining high survival and achieving improved growth. Maximising larval growth throughout hatchery culture will be critical in the economical production of a slow growing species such as KGW.

Previous studies indicate that the ability of larvae to digest prey varies with fish age and the production of digestive enzymes (Tucker, 1998). It may be possible that while larvae may be able to ingest *Artemia* (NI) at early stages, their digestive tract may not be developed enough to digest *Artemia* (NI). High survival of larvae fed *Artemia* (NI) from 51 dph (SL = 10.17 ± 0.75 (SE)) and 58 dph (SL = 10.61 ± 0.65 (SE)) indicates that KGW larvae are more competent to digest and utilise *Artemia* after they reach a size of 10.17mm SL. At this stage the development of the digestive system of KGW larvae may allow improved digestion and utilisation of *Artemia* (NI).

Although KGW larvae were able to ingest *Artemia* (N1) at 7.73mm SL in this trial, this was correlated with reduced survival. Alternatively the introduction of *Artemia* when larvae have attained approximately 10.0mm SL is recommended, as at this stage they appear to have an

improved ability to utilise this feed type. Further research on development of the digestive system in larvae of KGW would be required to provide a definitive explanation for this finding.

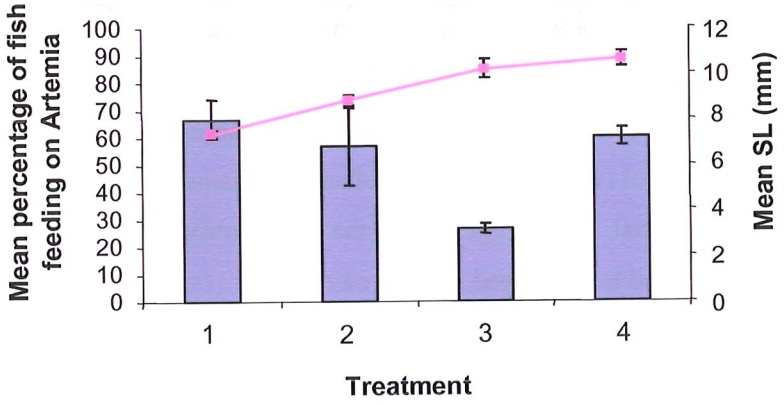


Figure 3.50 Percentage of KGW larvae feeding on *Artemia* (NI) 24 hours after the introduction of *Artemia* to each treatment (bar chart) and mean standard length (mm) (± SE) at time of commencement of each treatment (line).

Table 3.2 Mean standard lengths (mm), maximum mouth gape (mm) and predicted prey width that larvae are able to ingest (at 50 % mouth gape) for KGW larvae measured 24hrs after the commencement of *Artemia* (NI) feeding in each treatment.

Treatment	Standard length (mm)	± SE	Maximum mouth gape (mm)	± SE	Predicted prey width (mm) (50 % mouth gape)
1	7.73	0.17	0.40	0.04	0.20
2	8.74	0.24	0.52	0.03	0.26
3	9.36	0.59	0.78	0.06	0.39
4	11.62	0.84	0.80	0.06	0.40

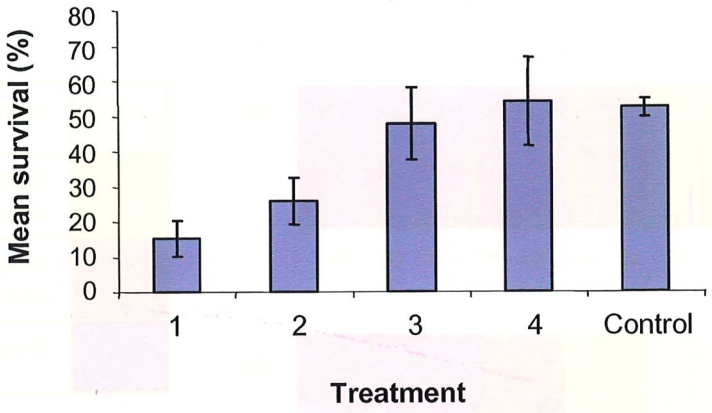


Figure 3.51 Mean percentage survival of KGW larvae in each treatment on completion of the trial (±SE).

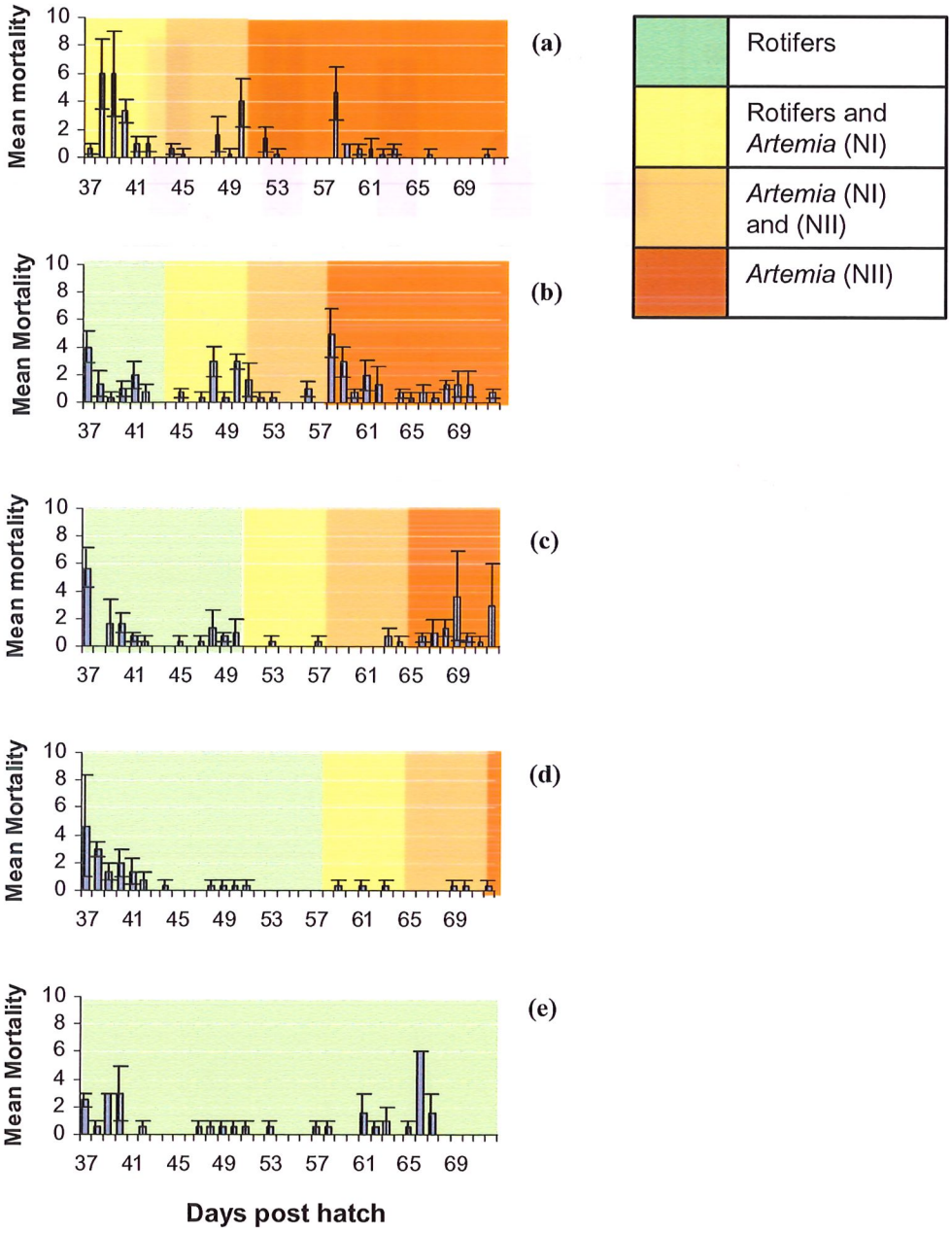


Figure 3.52 Mean number of mortalities (±SE) per day in (a.) Treatment 1 – NI *Artemia* fed at 37dph, (b.) Treatment 2 – NI *Artemia* fed at 44dph, (c.) Treatment 3 – NI *Artemia* fed from 51 dph, (d.) Treatment 4 – NI *Artemia* fed from 58dph and (e.) Control – rotifers fed only. Shaded regions indicate different feeding regimes as per legend.

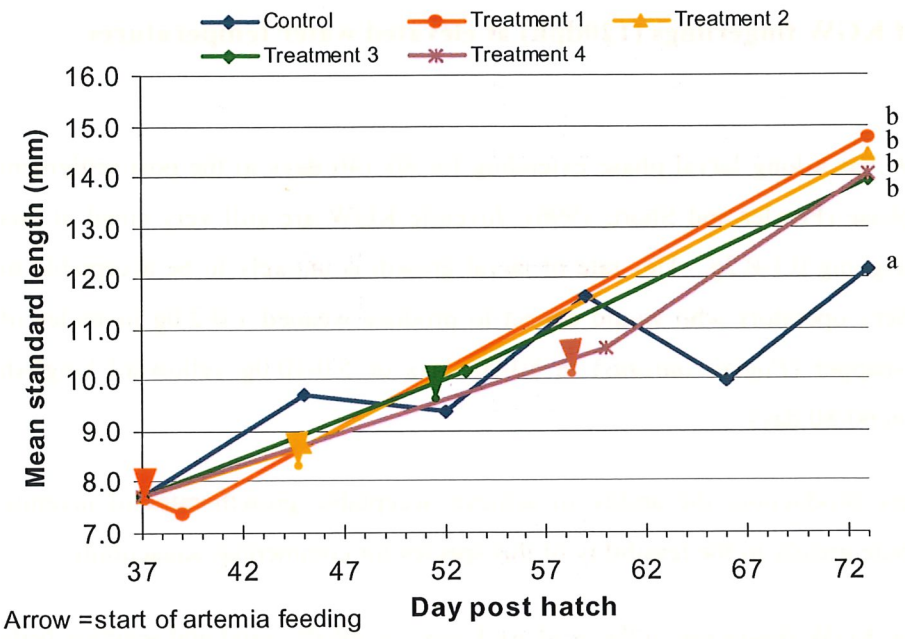


Figure 3.53 Mean standard length (mm) of rotifer fed KGW larvae transferred to *Artemia* (NI) at one-week intervals.

3.4 Growth of fingerlings

3.4.1 Growth of KGW fingerlings (120dph) at elevated water temperatures

Introduction

In the wild, KGW has a long larval phase extending for 80-146 days to the post settlement (early juvenile) phase (Fowler and Short, 1996). Juvenile KGW are still very small at this time, typically weighing 0.1-0.2g. This rate of larval growth is unlikely to be acceptable to commercial hatchery operators who would expect to produce weaned 1.0-2.0g juveniles of species such as snapper (*Pagrus auratus*) in 80-90 days or 5.0-10.0g yellowtail kingfish (*Seriola lalandi)* in 60-80 days.

Following hatchery production, the ability to achieve acceptable growth rates of juvenile KGW will contribute greatly to the feasibility of this species for commercial aquaculture.

In South Australia, KGW postlarvae in the wild are known to inhabit sand and seagrass beds in the sheltered, shallow waters of west coast bays and gulf waters (Bruce unpublished data as per Jones *et al* 1990). At this stage juveniles can be exposed to water temperatures that range from 14°C to 23°C (Fowler and Short, 1996). To investigate the ability to improve the growth of KGW fingerlings, a preliminary trial, assessing the growth of this species at elevated water temperatures, was conducted over a 70-day period. The water temperatures assessed in this study (18°C, 20°C, 22°C, 24°C and 26°C) incorporate those experienced by KGW fingerlings in the wild and extend beyond these temperatures to investigate whether growth rates achieved in the wild can be improved.

Aim 1

To investigate the optimum growth rate and survival of KGW fingerlings at a range of water temperatures from 18 °C to 26°C.

Method

Experimental set up

Twenty 120-dph fingerlings were stocked, per tank, into three replicate 30 L hemispherical fibreglass tanks randomly assigned to each of the five desired water temperatures (18°C,

20°C, 22°C, 24°C and 26°C). The standard length (mm) and weight (g) of each larva were recorded at stocking.

All tanks were set initially at 18°C and received flow through water at a rate of 8L/hr controlled by irrigation emitters. Over a period of 48 hours, the water temperatures were adjusted to the desired temperature for each treatment. Control of water temperature was achieved using a 200L bath, on which the tanks were mounted. A building automation system (BAS) controlling the delivery of hot, cold and ambient seawater to each water bath.

48 hours after stocking, mortalities (these occurred randomly throughout all treatments) were removed and replaced with fingerlings from a stock culture tank (1000L fibreglass culture tanks). Replacement fingerlings were acclimatised to the required temperature over a 24-hour period before being released in the treatment tank. The photoperiod was set at 12-hour light: 12-hour dark cycle.

Throughout the trial, fish in all tanks were fed enriched (DHA Selco®, Inve aquaculture) second stage *Artemia* nauplii (NII). Throughout the day each tank received particulate feed (ML 250 and ML 400, Nippai feeds®, Japan) fed *ad libitum* 6 times per day.

All fish were weighed and measured at two-week intervals. Tanks were fully cleaned during this process prior to the return of the fish.

Measuring fish

Fingerlings were netted from tanks and anaesthetised in a 10L bucket containing 20mg/L (ppm) benzocaine. Once anaesthetised, individual larvae were picked up in a strainer and excess water was removed by resting this on a paper towel. Fingerlings were transferred onto a pre-tared filter paper and the weight (g) was recorded using an electronic balance. Standard length (mm) was recorded from the snout to the caudal peduncle using digital callipers to ± 0.1mm. Measured larvae were placed in a recovery tub of seawater taken from their tank and returned to their respective tanks.

Data

ANOVA at 5 % significance level was used to test the difference in final wet weight (g) and specific growth rate (SGR) in temperature treatments over the 70-day period of the trial. Least significant difference (LSD) test was performed at 5% significance level to test for source of difference in final weights (g). Data was observed to be normally distributed using probability plot of residuals. Data was compiled in Microsoft Excel™ and all statistical analysis was performed in GenStat.

Results/discussion

Results from this trial can only be regarded as preliminary as several fingerlings jumped from tanks after stocking, irrespective of treatment. Other than this, no other mortality occurred for any treatment.

The final weight (g) of fingerlings was affected by temperature ($P_{(4,7)}$=0.038, Table A1.23 in Appendix 1). Fingerlings in temperature treatments 22°C to 26°C showed increased growth over a 70-day period from temperature treatments 18°C and 20°C (Figure 3.54) (Table A1.24 in Appendix 1). No difference was detected in ANOVA (Table A1.26 in Appendix) for the specific growth rate (SGR) ($P_{(4,7)}$=0.118) of treatments throughout the trial. The mean SGR values for all treatments ranged from 1.1 at 18°C to 1.83 at 26°C (Table 3.3). These SGR suggest that if these growth rates could be maintained, then time to market size (200g) at 18°C and 26°C would be between 630 days (21 months) and 380 days (12.5 months) respectively (market size is considered to be equal to the legal fishing limit of 30cm. Measurements conducted on captive fish of this length indicate that the corresponding weight is ~200g).

Results from this study show that KGW larvae (120 – 190 dph) can tolerate temperatures up to 26°C. Further research will be needed to determine the optimum and maximum water temperature for growth and survival during this stage of culture and through to market size. Ideally the effect of handling on fingerlings, would have been evaluated in this trial, however, limited availability of fish restricted the ability to assess this. This information is desired to fully evaluate treatment effects.

The growth of larvae in 22°C, 24°C and 26°C treatments is encouraging for efforts to reduce the time taken for hatchery rearing of juveniles. Shortening the duration of culture will reduce the cost of production and thus the sale price to on-growers.

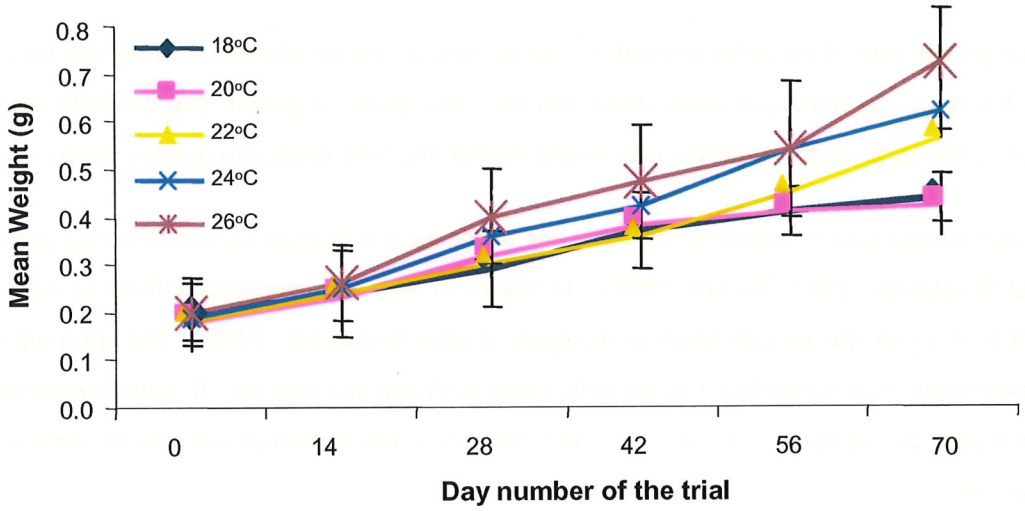


Figure 3.54 Mean wet weight (g) of KGW fingerlings cultured at elevated temperatures (18-26°C) over a 70-day period (±SE).

Table 3.3 Mean specific growth rate of KGW fingerlings cultured at water temperatures ranging from 18-26°C over a 70-day period.

Water temperature (°C)	Mean SGR (% inc. body weight per day)	Standard Deviation (SD)
18	1.11	0.49
20	1.21	0.96
22	1.62	0.83
24	1.69	0.64
26	1.83	0.80

3.4.2 Growth of KGW juveniles (146 and 205 dph) at elevated water temperatures

Introduction

The growth rate of juveniles through to market size is crucial when examining the feasibility of KGW as a commercial aquaculture species. Any gains in growth that could be achieved during the early stages of production would shorten the time needed to achieve market size.

Water temperature is widely accepted as one of the most important factors affecting growth of finfish (Barnes, 1963; Tucker, 1998). Increases in water temperature within the ecological limits of a fish species can result in increases in growth (Tucker, 1998). The optimum water temperature, that maximises fish growth, varies with age and species. If water temperature is optimised during grow-out, the time taken for fish to reach market size can be substantially reduced.

Preliminary studies (3.4.1 Growth of KGW fingerlings (120dph) at elevated water temperatures) conducted using early juvenile stage KGW indicate that this species can benefit from elevated water temperatures at this stage of their development. Approximately 150 KGW fingerlings were available after a spawning in 1998. These fish were used to conduct a preliminary study of on-growing of KGW at 20°C and 25°C. These temperatures were selected as they were within the range that could be tolerated by this species and could be maintained in commercial aquaculture production systems.

Trial 1

Aim 1

To examine the effect of elevated temperature (20°C and 25°C) on the growth and survival of KGW juveniles.

Aim 2

To document the food conversion rate (FCR) of KGW juveniles fed on a commercial barramundi diet in the temperature treatments investigated in this study (20°C and 25°C).

Method

Experimental set up

Three weeks prior to the commencement of the trial, two 1000L fibre reinforced plastic (FRP) tanks were stocked with 205 day post-hatch (dph) fingerlings at a density of 69 per tank. Temperature was recorded daily. The length (± 0.1 mm) and wet weight (± 0.1 g) of each fish was recorded prior to stocking. Tanks were set at 20°C using a BAS system with a 13-hour light: 11-hour dark cycle. Due to limitations in the number of fish available there was no replication.

Start of experiment (7/1/99)

At the beginning of the experiment, fish were randomly redistributed between the two tanks to ensure an even density and mean weight of fish. The length and weight of 20 random fish from each tank was recorded. Tanks were set at 20°C and one was increased by 1°C per day until 25°C was reached. Both tanks were supplied with a flow through of controlled temperature seawater. Their feeding regime (Table A1.26 in Appendix 1) was based on a commercial feeding table for European sea bream (*Sparus auratus*) (Petridis and Rogdakis, 1996). This was adjusted each fortnight based on the biomass in each tank and the previous fortnight's feed conversion rates (FCR). Feed was delivered to fish in each tank by a small automatic feeder (Fish Feeder, Japan) set to deliver the daily feed requirement over a number of individual feeds which varied throughout the trial.

Sampling

Each fortnight, fingerlings were netted into an aerated 20L bucket to which was added benzocaine at 20mg/L. Once sedated, fish were gently removed from the bucket and placed on a paper towel to remove excess water. The standard length (the length from the snout to the caudal peduncle) of the fish was recorded usually calipers (0-10cm) and a ruler (>10cm). Once measured, fish were placed into a tared container of seawater from the respective holding tank and wet weight was recorded (± 0.1g). Once fully recovered, fish were returned to their respective tank. For each tank the mean weight (g), mean length (mm), weight gain, feed per day (g), FCR and specific growth rates (SGR) were calculated on each sampling occasion.

Data

A t-test at 5% significance was used to test for significant difference in final weights (g) between the two temperature treatments. Data was observed to be normally distributed using probability plots of residuals. Data was compiled in Microsoft Excel™ and statistical analysis was performed in Statistix7.

Trial 2

At the completion of larval rearing in 1999, approximately 1200 fingerlings were available to conduct an on-growing trial including more water temperature treatments and replication.

Aim

To further investigate the growth and survival of KGW juveniles at 20°C, 23°C and 26°C.

Method

Experimental set up

The three desired water temperatures (20°C, 23°C, and 26°C) were allocated between six 600 L FRP tanks, providing 2 replicates per treatment. At stocking, all tanks were set at 22°C with a 13-hour light: 11 hour dark cycle. All tanks were fitted with 2 airlines and a constant flow rate of 720L/hr. A total of 1050 fingerlings at 146-day post hatch were netted from the stock culture tank and randomly allocated between the six experimental tanks (n = 175 per tank). The length and weight of 30 fingerlings were recorded prior to stocking the six tanks.

Start of the experiment

Tanks were adjusted to required temperatures 1-week post stocking (1°C/day). Feeding was set, based on feed tables (Table A1.27 in Appendix 1) established in the prior KGW juvenile growth trial, and adjusted every fortnight based on biomass and previous FCR in tanks. Feed was delivered to each tank using a belt feeder (AGK Technology).

A sub-sample of 40 fish from each tank was measured fortnightly, recording their weight (± 0.1g) and length ((± 0.1 mm). Water temperatures were recorded daily.

On day 89 of the trial, total mortality occurred in two tanks due to a computer malfunction that increased water temperatures up to 35°C. To compensate for this fingerlings were re-distributed between the 6 tanks (n = 110 fish per tank). From day 89 - 105 of the trial all

tanks were maintained at 23°C. Temperatures were readjusted to set temperatures on day 105. As the trial progressed it was observed that a wide size range was developing. On day 134 of the trial, the 25 smallest fingerlings were removed from each tank, representative of the grading process that occurs in commercial nurseries – i.e. removal of smaller individuals to maintain fish of approximately the same size in a tank. This is done in finfish primarily to assist with feeding regimes and to reduce cannibalism is some species. Each tank had an average mortality of 7 fish during the period 89 – 134dph, which resulted in a total density of 78 fish/tank after the removal of the 25 smallest fish/tank.

Measuring fish

A sub-sample of 40 fingerlings from each tank was transferred into an aerated 20L bucket containing benzocaine at 20mg/L. Once sedated, fish were gently removed from the bucket by hand and placed on paper towelling to removing excess water. The standard length of the fish was recorded with callipers (0-10cm) or a ruler (>10cm). Once measured, fish were placed into a pre-tared container of seawater from the respective tank and wet weight was recorded (± 0.01g). Once fully recovered, fish were returned to their respective tank.

Data

On each sampling occasion the mean weight, mean length, weight gain, feed per day, FCR and specific growth rates (SGR) were calculated for each tank. Analysis of variance (ANOVA) at 5% significance was used to test for significant difference in final weights (g) between temperature treatments. Data was observed to be normally distributed using probability plots of residuals. Data was compiled in Microsoft Excel™ and statistical analysis was performed in Statistix7.

Results and discussion (Trial 1 and Trial 2)

The FCR recorded (Table 3.4) at all water temperatures was excessive (range 2.91 – 5.10) and is attributed primarily to overfeeding throughout both trials. Although a feed table for sea bream was followed it was found that KGW are a difficult species to feed efficiently.

Survival in 20°C and 25°C treatments was 83.82% and 65.3% respectively (Table 3.4). Poor water quality (i.e. the presence of suspended solids in the water column) and periodic reduced dissolved oxygen (< 4ppm DO) in the 25°C treatment is thought to have contributed to the lower survival in this treatment. Under these conditions, uneaten food caused the water

quality to deteriorate more rapidly and quickly stressed the fish. Excluding mortalities due to computer malfunction, survival (Table 3.4) was high across all treatments (Trial 2 99.81%, 92.62% and 97.55% in 20, 23 and 26°C treatments respectively). These survival rates confirm that temperatures up to 26°C are within the ecological limits of KGW juveniles.

Results from Trial 1 (Figure 3.55) reveal that growth of juvenile KGW cultured at 25°C was higher ($P_{(2)(100)}<0.001$, Table A1.28 in Appendix 1) than that recorded at 20°C (Table 3.4). The SGR recorded for 1.9 – 2.17g fingerlings (205 dph), grown at 20°C and 25°C over a 308-day period, was 1.13 and 1.17 respectively. If these average growth rates are used as a crude estimate of the projected time for the stocked fingerlings (205 dph) to reach a market size of 200 g it would take 468 days (15.3 months) at 20°C and 452 days (14.7 months) at 25°C; a difference of only 2 weeks. These results will act as a guide to further experiments as an insufficient supply of fish restricted the ability to conduct a fully replicated trial.

Trial 2 results (Figure 3.56) starting with 146 dph fingerlings showed that no significant difference ($P_{(2,3)}=0.157$, Table A1.29 in Appendix 1) could be determined between any of the water-temperature treatments, although a trend of increasing growth rate with increasing water temperature was evident. Variations within treatments and a low number of replicates reduced the power of this test and the ability to detect a difference if one existed. The interval to market size (i.e. 200g) using the mean SGR values recorded suggest that it would take a further 405 days (13.2 months) at 20°C, 384 days (12.5 months) at 23°C, or 366 days (12.0 months) at 26°C (Table 3.4).

The high FCR values recorded in both trials will necessitate further research aimed at substantial reduction of feed used. Part of the problem may be attributed to the "flighty' behaviour of this species combined with their preference to feed low in the water column and off the bottom; and the relatively low feeding aggression shown in comparison to other commercial species (i.e. snapper, mulloway and yellowtail kingfish). These features combined make it difficult to assess the feeding of this species and any future work needs to focus on an improved strategy to maximise feeding efficiency. Another contributing factor may be the nutrition provided by the feed selected. No previous dedicated studies have been conducted to define the nutritional requirements of KGW. As such the diet used may not be optimal for this species.

The substantially higher SGR of fish less than 40.0g compared with that of fish greater than 40.0g is of interest (Table 3.4). In trial 1 the SGR for fish less than 40.0g were 1.39 and 1.84

at 20°C and 25°C respectively. For fish greater than 40.0g these SGR values dropped to 0.54 and 0.50 in 20°C and 25°C treatments respectively, representing only 38.8% and 27.1% of the growth rate achieved up to 40.0g for the respective treatments. In Trial 2 the SGR for fish grown up to 40.0g were 1.44, 1.76 and 1.91 at 20°C, 23°C and 26°C respectively. For fish greater than 40.0g these SGR values dropped to 0.67, 0.80 and 0.83 in 20°C, 23°C and 26°C treatments respectively, representing only 46.3%, 45.4% and 43.5% of the growth rate achieved up to 40.0g for the three water-temperature treatments.

The reduction in SGR values for KGW from 40.0g, observed in both trials, is difficult to explain. It is accepted that an increase in fish weight will be accompanied by a decrease in SGR even under constant environmental conditions with adequate feed availability (Laird and Needham, 1988; Petridis and Rogdakis, 1996). As an example, the SGR of juvenile halibut (*Hippoglossus hippoglossus*) has been shown to decline rapidly with increasing size. A preference for colder water temperatures (i.e. optimum 11°C) with increasing size has also been demonstrated with this species (Aune *et al*, 1997). It is recognised that fish growth may occur in "stanzas" that have different SGR values that show a reduction throughout the growth process (Laird and Needham, 1988). It may be that the decline in SGR of KGW may reflect changing nutritional or environmental requirements for fish at different stages of development. Further research will be required to investigate methods to improve SGR of KGW as growth progresses beyond 40.0g.

Ideally the effect of handling on juveniles would have been evaluated in these trials, however, limited availability of fish restricted the ability to assess this. This information is desired to fully evaluate treatment effects.

Table 3.4 Summary of FCR and SGR data for Trial 1 and Trial 2. (Note: Data for Trial 1 is for individual tanks while for Trial 2 data is the mean of 2 tanks per treatment).

	Trial 1 (308 days)		Trial 2 (267 days)		
	20°C	25°C	20°C	23°C	26°C
Initial weight	1.90	2.17	1.43	1.43	1.43
Final Weight	63.81	82.96	44.49	53.88	64.13
Survival	83.8%	65.3%	99.8%	92.6%	97.6%
FCR	3.82	5.10	2.91	3.01	3.67
SGR (%/day)	1.13	1.17	1.32	1.39	1.46
Projected days to 200 g	468	452	405	384	366
Projected months to 200 g	15.3	14.7	13.2	12.5	12.0
SGR until 40 g	1.39	1.84	1.44	1.76	1.91
SGR after 40 g	0.54	0.50	0.67	0.80	0.83
% of SGR until 40g	38.8%	27.1%	46.3%	45.4%	43.5%

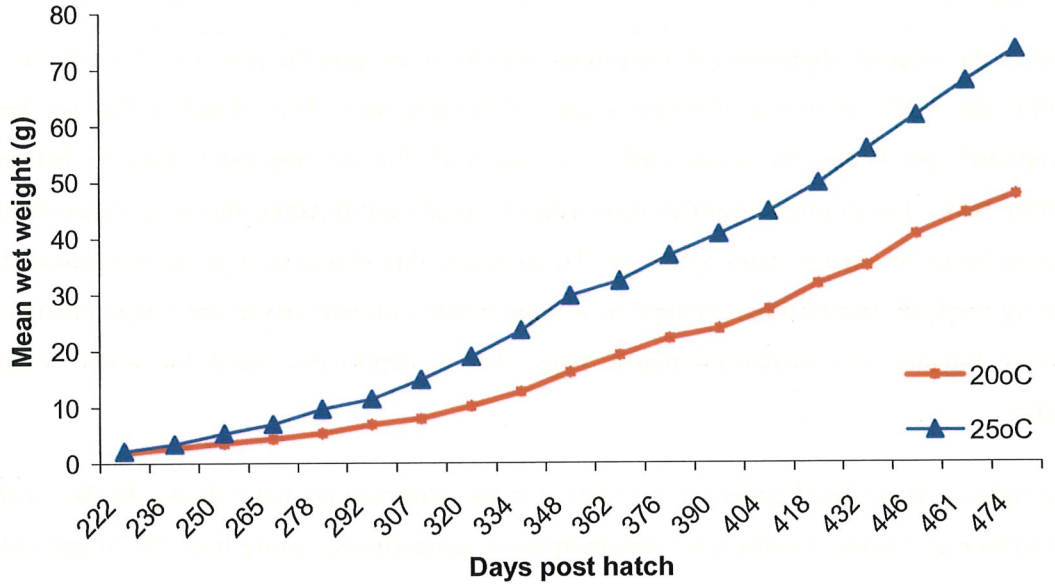


Figure 3.55 Mean wet weight (g) of KGW fingerlings grown at 20 and 25°C.

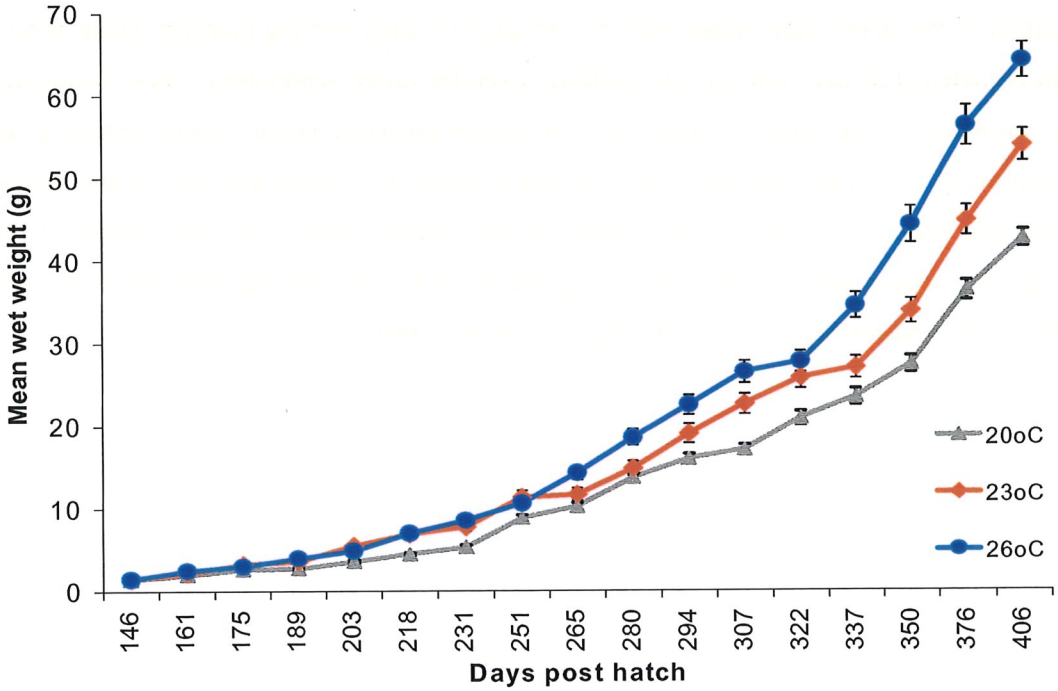


Figure 3.56 Mean wet weight (g) of KGW fingerlings grown at 20, 23 and 26°C (± SE).

Chapter 4 Behaviour of larvae in the water column

One of the original objectives of this project was to investigate the position of KGW larvae within the water column at different stages of development. This objective has not been completed specifically, as larvae used in rearing trials did not respond to transfer between rearing tanks. For example, transfer from 1000 L larval culture tanks into 30 L experimental rearing tanks resulted in high mortality. To complete this objective it is recommended that rearing methods require improvement to a stage where cultured larvae are robust enough to survive transfer into suspended plastic bags (3-6 m depth) envisaged for water column studies.

Throughout larval development in all rearing trials, observations have shown KGW inhabit the surface and sides of tanks until metamorphosis, generally occurring from 50-70 dph (14 – 20 mm SL) at water temperature of 20 – 24°C. A distinct change to a mostly bottom-dwelling behaviour was coincident with this change in morphology.

These observations agree well with the accepted life cycle for this species in which larvae remain planktonic during an extended development stage that occurs over winter months. The duration of the larval stage ranges from 67- 80 days for early settling juveniles (June-July) to approximately 120 days old for later-settling juveniles (after September). These differences are attributed to the date of spawning with earlier-spawned larvae metamorphosing and recruiting at a younger age than later spawned larvae. It is expected that metamorphosis would precede or coincide with the advection into nursery areas. This recruitment of post larvae into shallow sub-tidal beds of *Zostera* and *Heterozostera* spp has been shown to occur between July-November each year (Fowler and Short, 1996).

Chapter 5 Potential of KGW for commercial aquaculture

The most compelling reason to pursue research to develop commercial aquaculture of KGW is the established strong market status of this species. KGW is the second most valuable fish species in temperate Australia after Southern Bluefin Tuna. Fillets of KGW retail for up to $45/kg and fishermen receive $15 - $18/kg for whole fish (2003 prices). Other species with aquaculture potential in South Australia that receive lower prices include yellowtail kingfish ($7.00 - $12.00/kg) mulloway ($7.00 - $7.50/kg) and snapper ($7.00 - $10.00/kg) for gilled and gutted fish. These fish species have significantly faster growth rates compared to KGW (Table 5.1).

Table 5.1 Comparison of market size, time to market and wholesale price of aquaculture finfish species (Prices adjusted to whole fish assuming 10% loss during gilling and gutting).

Species	Market size (g)	Time to market (months)	Wholesale price ($/kg whole fish)
King George whiting	>170g	24 - 30	16.50 – 19.80
Yellowtail kingfish	> 3 kg > 5 kg	13 - 15 25 - 27	7.00 – 12.00
Snapper	>400 g	16 - 20	7.00 – 10.00
Mulloway	> 400 g	14 - 16	7.00 – 7.50

Currently the annual fishery production of KGW ranges from approximately 500 – 800 tonnes. Supply varies seasonally with limited amounts available over summer. This unpredictable supply often causes the price to fluctuate dramatically. The combined result makes it difficult for restaurants to maintain KGW as a menu item. Food industry responses confirm that KGW has high consumer appreciation and demand that would increase if a consistent supply of good quality KGW could be delivered year round. A consistent supply, allowing restaurants to incorporate KGW into their regular menus, could extend the current market, particularly in New South Wales and Victoria.

Comparison of sales of 253 tonnes of KGW through major national fish markets (Figure 5.1) in an indicative year (1998/99) shows that the amount sold in Adelaide (51.96%) was slightly higher than that sold in Melbourne (46.48%), with little sold in Sydney. The higher populations of these states suggest that, with a reliable supply, these markets could be expanded significantly. If these percentages are extrapolated over an average national catch of 700 tonnes pa, the Adelaide market would account for 363 tonnes pa, Melbourne 325 tonnes

pa and Sydney only 11 tonnes pa. The very small figure for Sydney suggests that supply is limited and adequate prices are received for fish sent to easily accessible markets in Adelaide and Melbourne.

A simplistic estimate of the potential market for KGW may be provided using these figures to estimate possible consumption rates for KGW. In Adelaide (1 million people) the consumption rate equates to 0.36 kg/person pa. If this level of consumption could be generated in larger markets (ie. Sydney 4.5 million and Melbourne 3.0 million people) through increased and more consistent supply, it may be possible that a national market in the order of 3000 tonnes pa could be achieved, valued at $46 million based on farm gate price of $15.00/kg.

Throughout the research project it has been demonstrated that KGW beyond the juvenile stage are durable under culture conditions and are not cannibalistic at any stage of development. In addition, KGW are easily handled and have shown minimal incidence of disease or the parasitic infestations that hinder the production of several species being cultured in Australia such as fluke (*Benedinia seriolae* and *Xuzapta seriolae*) infestations of yellowtail kingfish and gill amoeba (*Paramoeba*) of Atlantic salmon. These features should provide benefits for husbandry operations required during hatchery and on-growing to market size.

KGW are widely distributed across southern temperate waters of Australia from Port Jackson in New South Wales to Jurien Bay in Western Australia, including northern Tasmania (Kailola *et al* 1993). As such, KGW present a species with culture potential over a wide range of locations, if suitable production methods and culture systems can be developed. It is expected that KGW will be more amenable to pond and tank-farming systems and research has concentrated on growth trials using these methods. It is not envisaged that sea-cage farming will be practical for this species as the long cylindrical shape of juveniles in particular will require small mesh sizes to retain fish. The bottom-dwelling habit of KGW will also make feeding in sea cages difficult to manage. Sea cage systems appear more suited to species such as southern bluefin tuna, Atlantic salmon, snapper and yellowtail kingfish.

Although this species has some features that provide advantages for aquaculture, several challengers were encountered in this study that affect the commercialisation potential of KGW.

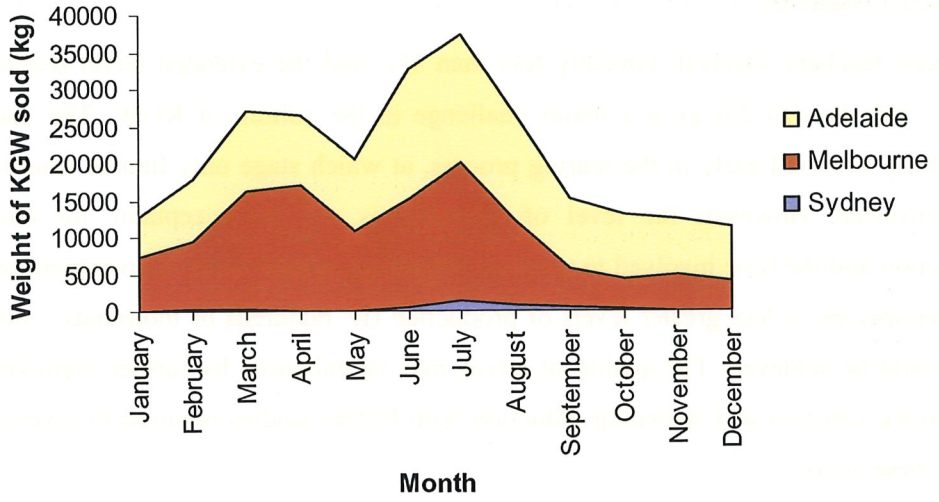


Figure 5.1 Volume (kg) of KGW sold in Safcol fish market (Adelaide), Sydney and Melbourne fish markets 1998/99 (Safcol fish market records; www.chsmith.com.au/fish-prices/index.htm; www.sydneyfishmarket.com.au).

Egg supply

A significant problem encountered in this study was an inability to achieve a regular supply of good quality eggs. Results from spawning trials indicate that KGW do not undergo reliable voluntary spawning in captivity in either natural or manipulated environments. Female broodstock exhibited spawning dysfunction throughout the project, with typically low spawning fractions (number of fish participating in spawning). Final oocyte maturation (FOM) was also unpredictable or absent in spawning fish, resulting in erratic spawning and a fluctuating fertilisation rate between 0 to 90% (typically less than 50%).

To overcome these problems and achieve regular egg production, hormone induction methods should be employed. Trials throughout this project have demonstrated that induction procedures using the hormones LHRHa and Ovaprim® are successful in advancing the reproductive development and inducing ovulation and spawning of captive broodfish during both the natural and manipulated seasons. Preliminary investigations into the optimum hormone dose and latency period (for broodfish being induced via injection) have indicated that doses between 10-20µg/kg bw (for both LHRHa and Ovaprim®), with a latency period between 50-54 hours, are most successful. However, the quality and quantity of eggs produced, following this regime, is not yet sufficient for commercial operations requiring millions of eggs on specific days and further studies are required to achieve this.

Hatchery Production

The poor hatchery survival, typically less than 1%, and the extended larval rearing period (120 – 140 days to 2.0 g) is a major challenge in the culture of KGW. The majority of mortalities occurred early in the rearing process, at which stage only limited resources have been invested. However, the level of mortality is not yet acceptable for commercial production and the time involved to produce fingerlings would make them expensive relative to other species, unless greater levels of production (ie. hundreds of thousands – millions of fish) could be achieved. The quality of larvae may be enhanced by further improvements to broodstock nutrition and spawning induction with further studies required to investigate and refine these areas.

Growth Rate

The growth rate of KGW juveniles through to market size is crucial when examining the feasibility of KGW as a commercial aquaculture species. The extended larval period and slow growth rate achieved is a major challenge for the culture of KGW. In the wild, KGW have a long larval phase extending for approximately 80-150 days to the post-settlement phase (Fowler and Short, 1996). Trials throughout this study have indicated that early growth can be accelerated by maintaining elevated water temperatures (20 – 26°C) and attention to feeding regimes. However, this growth is still slow in comparison to other species (i.e. snapper and yellowtail kingfish). The growth rates achieved during larval production trials are thought to have been compromised by use of sub-optimal quality larvae and limited numbers restricting our ability to grade fish and remove runts from the system. With cannibalistic species there is a tendency for ongoing removal of poorly performing larvae and fingerlings during culture, through attack during rearing, and grading undertaken by hatchery operators, so that surviving fish generally represent a high-performing cohort. To date this has not been the case with KGW, as all growth assessments have relied upon all available fingerlings, the supply of which has been limited.

Results from trials indicate that it is possible to accelerate the growth of KGW by increasing the water temperature. This would be best achieved using recirculating aquaculture systems in which a controlled environment can be maintained over the entire production cycle. To obtain a clearer picture of growth potential of KGW, further studies would be required to achieve progress to the point where large numbers of high quality larvae can be produced routinely. If

this could be achieved then better assessment of growth potential of top grade fish could be undertaken.

Given that this species is expected to command and sustain a price in excess of $15.00/kg, it is helpful to undertake simple analysis of various production cost scenarios (Table 5.2). From this, it is expected that a production cost of less than $13.50/kg will be difficult to achieve with KGW. Production costs in this order can only be predicted if the following performance targets for this species can be achieved;

- Market size achieved is 220g plus.
- Time to market reduced to 18 months or less.
- Fingerling cost less that $0.40c/unit.

None of these requirements have been achieved in the current study and it is likely that they could only be attained after the combined problems of egg supply and larval rearing are overcome and a sustained broodstock selection adopted to systematically improve growth rates. This will require commitment to a long-term moderately funded research program that will be difficult to justify based on outcomes achieved to date.

Table 5.2 Simple scenario to estimate production cost for on-growing of King George whiting in 100t recirculating aquaculture system.

Assumptions	
Production (tonnes)	100
Months to grow	18
Production cycle (years)	1.5
Survival rate	90%
Market size (g)	220

		Production cycle costs	
Capital		$1,000	% of Costs
System $1000/t	10		
System capital ($million)	1		
Land and services ($million)	0.5		
Capital cost ($million)	1.5		
Interest (% pa)	8%		
Interest ($1000 pa)	120		
Depreciation (% pa)	10%		
Interest		180	13.34%
Depreciation		225	16.68%
Labour			
FTE/tonne	20		
FTE's	5		
$1000/FTE (inc on-cost)	40		
Labour		300	22.24%
Operating Costs			
Power			
$1000/100 tonne pa	75		
Power		113	8.34%
Maintenance (% capital pa)	5%		
Maintenance		75	5.56%
Feed			
FRC	1.2		
Feed ($/kg)	1.7		
Feed (Tonnes)	120		
Feed		204	15.12%
Fingerlings			
Cost $/unit	0.5		
No. needed/kg	5.05		
No. needed pa (x1000)	505		
Cost $/kg Production	2.53		
Fingerlings		253	18.72%
Total Production Costs $		1,349.03	100%
Total Production Cost $/kg		13.49	

Benefits

1. Essential information on the challenges associated with controlled egg production, and the culture of KGW in general, have been provided.

2. Fundamental biological information on the reproduction and development of KGW eggs and larvae has been documented. This information is beneficial for the culture of this species and will assist further fisheries research.

3. Identification and characterisation of the degeneration of post-ovulatory follicles in KGW will assist fisheries scientists to identify the time of spawning, spawning frequency and, consequently, estimates of spawning biomass. This information is crucial in developing effective management practices that are necessary to ensure the sustainability of the fishery.

4. Identification and documentation of the disease *Exophiala salmonis*. This disease has the potential to have a significant effect on captive KGW stocks and was previously not known to have occurred in this species.

5. Egg incubation and larval rearing trials have provided information on the ecological limits of this species at different stages in their development. Trials have indicated that environmental factors (i.e. temperature and salinity) can impact the growth and survival of this species. This information will benefit further research and commercialisation of the species, allowing environmental conditions to be manipulated to maximise growth.

6. Preliminary studies investigating the grow-out of juveniles have provided information that suggests that the growth of juveniles can be accelerated at water temperatures up to 26°C. Further research will be required to achieve commercially-viable growth rates and FCR's.

7. Completion of this project has provided information to allow the challenges associated with commercial aquaculture of KGW to be more accurately assessed.

8. The production of the Coastal Finfish Hatchery Manual: King George Whiting (*Sillaginodes punctata*) has documented information, which was collated throughout this project. The information presented in this manual provides people, with an

interest in marine finfish hatchery operations, with preliminary results of research trials conducted to date and protocols developed throughout this project. It aims to cover the fundamental aspects of hatchery production for marine finfish in general using KGW as the focus species.

Further Development

The growth rate of KGW juveniles through to market size is crucial when examining the feasibility of KGW as a commercial aquaculture species. To gain insight into the economic viability of KGW grow-out in commercial systems, 1g fingerlings were stocked in a saline pond at Port Augusta, in tanks using recirculating ground water at Posaqua, Tickera and at the Bedford Saline Ground Water Interception Scheme at Cooke Plains, run by the Coorong District Council. Growth data was collected from each site monthly and will be collated to determine realistic growth rates and the economic viability of KGW grow-out.

Based on the results of this current project KGW is not a viable species for commercialisation at this stage. To improve this situation it is suggested that a number of key areas of investigation should be followed, including;

1. Further improvement in methods to control supply and quality of eggs, particularly focusing on improving the methods used for hormone induced spawning and broodstock nutrition.

2. Improved hatchery survival. As part of this, assessing the different levels of lipid and protein that could be supplied to live feeds and passed on to larvae, should be investigated. The morphological and physiological development of the gastrointestinal tract of KGW larvae should be investigated to identify timing of formation of major organs and enzyme systems. This information will assist in identifying suitable feeds and nutrition to be provided at specific times in the development of KGW larvae.

3. Grow-out evaluations should be undertaken, once large numbers of fingerlings have been produced (>100,000), to allow selection of the best performing fish (top 20-30%) at an early age (as is the case with more cannibalistic species).

4. Ongoing selection of broodstock for improved growth performance.

These research objectives will require a medium to long-term commitment of resources.

Planned outcomes

As a result of this project, with assistance from the "Farmed Seafood Inititative" of the South Australian Government, a Coastal Finfish Hatchery Manual (Coastal Finfish Hatchery Manual: King George Whiting *(Sillaginodes punctata)* by Jane Ham and Wayne Hutchinson) was produced (refer to Appendix 2). The hatchery manual presents protocols and incorporates preliminary results of research trials conducted to date. It aims to cover the fundamental aspects of hatchery production for marine finfish, in general, using KGW as the focus species. The areas covered in this manual include: broodstock systems, broodstock husbandry, egg biology, controlled egg production, larval rearing including live feed production, nursery and subsequent grow-out, health and diseases.

The Coastal Finfish Hatchery Manual: King George whiting *(Sillaginodes punctata)* is available as both an easy to follow interactive CD ($49.50) and in hard copy ($99) (all prices include GST and postage). Please contact SARDI Aquatic Sciences' library to obtain a copy. Ph: (08) 8200 2423; Fax: (08) 8200 2481; E-mail: bennett.suzanne@saugov.sa.gov.au; Postal address: PO Box 120 Henley Beach, SA 5022.

Conclusion

This project 'Spawning and larval rearing research on King George whiting (*Sillaginodes punctata*) relevant to aquaculture and fisheries biology,' highlighted the challenges associated with the production of KGW. The main achievements were:

1. documenting fundamental information on the reproduction and development of KGW eggs and larvae,
2. identification and characterisation of the degeneration of post-ovulatory follicles in KGW that will assist fisheries scientists to identify the time of spawning, spawning frequency and consequently estimates of spawning biomass,
3. identification and documentation of the disease *Exophiala salmonis*,
4. investigating the ecological limits of this species at different stages in their development through egg incubation and larval rearing trials,
5. investigating the growth of KGW juveniles at elevated water temperatures and
6. providing information to allow the challenges associated with culture of KGW to be more accurately assessed.

The main problems encountered were:

1. controlled supply of good quality eggs from broodstock,
2. larval survival in the hatchery and
3. the slow growth of KGW throughout both the larval and grow out periods.

Species being commercially cultured generally exhibit characteristics that allow relatively routine hatchery-rearing, if appropriate hatchery procedures are followed. For example, species such as snapper, mulloway and yellowtail kingfish broodstock can be induced to spawn in captivity without too much intervention. These species also provide good larval survival rates (>10% - 50%) and fast growth rates (ie. to 1- 2 g in 60 – 80 days) that allow relatively cost-effective production of commercial numbers of fingerlings (ie. hundreds of thousands). This level of production allows selection of better performing fish for supply to on-growers. Although a good market with room for expansion exists for KGW this species

does not present key features that make it conducive to economical hatchery production and on-growing. This makes it difficult to justify ongoing research for aquaculture development of this species, although this work will need to be continued if stock enhancement is considered as a fishery management option at any stage in the future.

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Appendix 1

Table A1.1 Two-way analysis of variance with interaction at 5% significance level to test the difference in the hatching success of KGW eggs at different temperatures (20, 22, 24, 26 and 28°C) and salinities (30, 35, 40, 45, 50 and 55) (Partington, 1999).

Source	DF	Sum of squares	Mean square	F Value	P value
Temperature	4	74003.49	18500.87	301.37	<0.01
Salinity	5	3726.56	745.31	12.14	<0.01
Temp*salinity	20	4063.37	203.17	3.29	<0.01
Residual					
Replicate	3	660.17	220.06	3.58	0.02
Replicate*temp	11	2222.21	202.02	3.29	<0.01
Error	70	4297.30	61.39		
Total	113	91264.41			

Table A1.2 Two-way analysis of variance with interaction at 5% significance level to test the difference in the hatching success of KGW eggs at different temperatures (16, 19, 22, 25 and 28°C) and salinities (25, 30, 35, 40, 45 and 50) (Partington, 1999).

Source	DF	Sum of squares	Mean square	F value	P value
Temperature	4	77403.98	19351.00	318.39	<0.01
Salinity	5	11185.98	2237.20	36.81	<0.01
Temp*Salinity	20	17166.66	858.33	14.12	<0.01
Residual	75	4558.30	60.78		
Replicate	3	143.42	47.81	0.79	0.51
Rep*temp	12	555.45	46.29	0.76	0.69
Total	119	111013.80			

Table A1.3 Staining procedure used for KGW larvae (modified from Potthoff, 1984).

Solution	Time in solution for larvae <10mm	Time in solution for larvae >10mm < 80mm
Fixation 10% formalin	2 days - 1 year	2 days - 1 year
Dehydration 1. 50% distilled water 50% ethanol (95%) 2. Absolute ethanol 95% ethanol may be substituted	 1 day 1 day	 2 days 2 days
Staining cartilage 100mL solution of 70mL absolute ethanol 30mL acetic acid 20mg alcian blue	1 day	1 day
Neutralisation Saturated sodium borate solution	1/2 day	1/2 day
Bleaching 100mL solution of 15 mL 3% H_2O_2 85 mL 1% KOH	Not necessary	< 20 minutes
Trypsin digestion 100 mL solution of 35 mL saturated sodium borate 65 mL distilled H_2O, 1g trypsin powder	1 day (or until 60% clear)	3-4 days (or until 60% clear)
Staining bone 1% KOH solution with alizarin red (add alizarin red until solution turns deep purple)	1 day	1 day
Destaining 100 mL solution of 35 mL saturated sodium borate 65 mL distilled H_2O 1g trypsin powder	2 days	2+ days until the solution remains unstained and the specimen is clear
Preservation 1. 30% glycerin 70% of 1% KOH 2. 60% glycerin 40% of 1% KOH 3. 100 % glycerin with thymol as a final preservative	 1 week 1 week remain in solution	 2 weeks 2 weeks remain in solution

Table A1.4 Staining procedure used for KGW juveniles (modified from Potthoff, 1984).

Solution	Time in solution for larvae > 100mm
Fixation 10% formalin	5 days, flesh removed on one side
Dehydration 1. 50% distilled water 50% ethanol (95%) 2. Absolute ethanol 95% ethanol may be substituted	 5 days 7 days
Staining cartilage 100mL solution of 60mL absolute ethanol 40mL acetic acid 30mg alcian blue	1.5 days
Neutralisation Saturated sodium borate solution	2 days (one intermediate change)
Bleaching 100mL solution of 15 mL 3% H_2O_2 85 mL 1% KOH	1 hour
Trypsin digestion 100 mL solution of 35 mL saturated sodium borate 65 mL distilled H_2O, 1g trypsin powder	12 days (solution changed after 10 days)
Staining bone 1% KOH solution with alizarin red (add alizarin red until solution turns deep purple)	2 days
Destaining 100 mL solution of 35 mL saturated sodium borate 65 mL distilled H_2O 1g trypsin powder	30 days (solution changed every 10 days)
Preservation 1. 60% glycerin 40% of 1% KOH 2. 100 % glycerin with thymol as a final preservative	4 weeks Final preservative

Table A1.5 Two-sample t-test at 5% significance level to test the difference in the mouth gape (mm) of KGW larvae (4dph) at hatch in different salinities (30 and 35).

t statistic	0.93
d.f.	18
P	0.36

Table A1.6 Analysis of variance at 5% significance level to test the difference in the hatching success of KGW larvae in salinity treatments (30, 35, 40, 45, 50 and 55).

Source	Sum of squares	DF	Mean Square	F-ratio	P
Salinity	2341.65	5	468.33	1.01	0.45
Error	5557.45	12	463.12		
Total	7899.10	17			

Table A1.7 Analysis of variance at 5% significance level to test the difference in the yolk sac volume of KGW larvae at hatch in different salinities (30, 35, 40, 45, 50 and 55).

Source	Sum of squares	DF	Mean Square	F-ratio	P
Tank stratum Salinity	1.886E+16	5	3.773E+15	1.46	0.28
Residual	2.851E+16	11	2.591E+15	2.44	
Tank.Salinity. *Units*stratum	1.360E+17	128	1.062E+15		
Total	1.833E+17	144			

Table A1.8 Analysis of variance at 5% significance level to test the difference in the yolk sac volume of KGW larvae at 1DPH in difference salinities (30, 35, 40, 45, 50 and 55pt).

Source	Sum of squares	DF	Mean Square	F-ratio	P
Tank stratum Salinity	5.741E+15	5	1.148E+15	2.11	0.14
Residual	5.994E+15	11	5.449E+14	1.76	
Tank.Salinity. *units*.stratum	4.175E+16	135	3.093E+14		
Total	5.349E+16	151			

Table A1.9 Analysis of variance at 5% significance level to test the difference in the yolk sac volume of KGW larvae at 2 DPH in different salinities (30, 35, 40, 45, 50 and 55).

Source	Sum of squares	DF	Mean Square	F-ratio	P
Tank stratum Salinity	8.919E+14	5	1.784E+14	2.70	0.08
Residual	7.271E+14	11	6.610E+13	1.25	
Tank.Salinity. *units*.stratum	6.415E+15	121	5.301E+13		
Total	8.034E+15	137			

Table A1.10 Analysis of variance at 5% significance level to test the difference in the yolk sac volume of KGW larvae at 3 DPH in different salinities (30, 35, 40, 45, 50 and 55).

Source	Sum of squares	DF	Mean Square	F-ratio	P
Tank stratum Salinity	7.936E+13	5	1.587E+13	0.82	0.59
Residual	9.719E+13	5	1.944E+13	4.26	
Tank.Salinity. *units*.stratum	3.743E+14	82	4.564E+12		
Total	5.508E+14	92			

Table A1.11 Two-sample t-test at 5% significance level to test the difference in the yolk sac volume of KGW larvae at 4 DPH in different salinities (30 and 35).

t statistic	-0.00
d.f	35
P value	0.99

Table A1.12 Analysis of variance at 5% significance level to test the difference in the total length (mm) of KGW larvae at hatch in different salinities (30, 35, 40, 45, 50 and 55).

Source	Sum of squares	DF	Mean Square	F-ratio	P
Tank stratum Salinity	632522	5	126504	1.09	0.42
Residual	1275627	11	115966	3.01	
Tank.Salinity. *units*.stratum	4933331	128	38542		
Total	6841480	144			

Table A1.13 Analysis of variance at 5% significance level to test the difference in the total length (mm) of KGW larvae at 1 DPH in different salinities (30, 35, 40, 45, 50 and 55).

Source	Sum of squares	DF	Mean Square	F-ratio	P
Tank stratum Salinity	2405968	5	481194	2.31	0.11
Residual	2287625	11	207966	4.80	
Tank.Salinity. *units*.stratum	5801155	134	43292		
Total	10494749	150			

Table A1.14 Analysis of variance at 5% significance level to test the difference in the total length (mm) of KGW larvae at 2 DPH in different salinities (30, 35, 40, 45, 50 and 55).

Source	Sum of squares	DF	Mean Square	F-ratio	P
Tank stratum Salinity	636622	5	127324	0.61	0.69
Residual	2291275	11	208298	3.33	
Tank.Salinity. *units*.stratum	7557451	121	62458		
Total	10485348	137			

Table A1.15 Analysis of variance at 5% significance level to test the difference in the total length (mm) of KGW larvae at 3 DPH in different salinities (30, 35, 40, 45, 50 and 55).

Source	Sum of squares	DF	Mean Square	F-ratio	P
Tank stratum Salinity	1229336	5	245867	1.07	0.47
Residual	1152742	5	230548	3.37	
Tank.Salinity .*units*.stratum	5611602	82	68434		
Total	7993680	92			

Table A1.16 Two-sample t-test at 5% significance level to test the difference in the total length (mm) of KGW larvae at 4 DPH in different salinities (30 and 35).

t statistic	0.82
d.f.	35
P value	0.42

Table A1.17 Two-sample t-test at 5% significance level to test the difference in the total length (mm) of KGW larvae at 6 DPH in different salinities (30 and 35).

t statistic	0.41
d.f.	11
P value	0.69

Table A1.18 Analysis of variance at 5% significance level testing the difference in the percentage of KGW larvae feeding on *Artemia* nauplii 24 hours after the introduction of *Artemia*.

Source	Sum of squares	DF	Mean Square	F-ratio	P
Treatment (A) Tank (B)	2825.00	3	941.67	1.40	0.31
A*B	5400.00	8	675.00		
Total	8225.00	11			

Table A1.19 Analysis of variance at 5% significance level testing the difference between survival (%) of KGW larvae in each treatment on completion of the trial.

Source	Sum of Squares	DF	Mean Square	F-ratio	P
Treatment (A) Tank (B)	5234.30	4	1308.58	10.87	<0.01
A*B	1083.12	9	120.346		
Total	6317.42	13			

Table A1.20 Tukey-Kramer HSD comparisons for all pairs of survival (%) of KGW larvae on completion of the trial (homogenous groupings are indicated by the same notation).

Treatment	Homogenous groupings
1	A
2	A
3	B
Control	B
4	C

Table A1.21 Analysis of variance at 5% significance level in standard length (mm) of KGW larvae between treatments on completion of the trial.

Source	Sum of squares	DF	Mean Square	F-ratio	P
Tank stratum Treatment	84.69	4	21.17	10.34	<0.01
Residual	18.42	9	2.05	1.00	
Tank.Treatment.*Units*. Stratum	246.37	120	2.05		
Total	349.49	133			

Table A1.22 Tukey-Kramer HSD comparisons for all pairs of standard length (mm) of KGW larvae between treatments on completion of the trial (homogenous groupings are indicated by the same notation).

Treatment	Homogenous groupings
1	A
2	A
3	A
4	A
Control	B

Table A1.23 Analysis of variance at 5% significance of mean weights (g) of KGW fingerlings cultured at elevated temperatures (18-26°).

Source	Sum of Squares	DF	Mean square	F-ratio	P
Tank stratum Temperature	0.82	4	0.21	4.65	0.04
Residual	0.31	7	0.04	1.60	
Tank.Temperature.*Units* stratum	1.91	69	0.03		
Total	3.04	80			

Table A1.24 LSD comparisons for all pairs of mean weight (g) of KGW fingerlings cultured at elevated temperatures (18-26°C) (homogenous groupings are indicated by the same notation).

Temperature (°C)	Homogenous groupings		
18	A		
20	A	B	
22		B	
24		B	C
26			C

Table A1.25 Analysis of variance at 5% significance level of mean SGR of KGW larvae in temperature treatments (18°C-26°C) over a 70-day period.

Source	Sum of Squares	DF	Mean square	F-ratio	P
Tank Stratum Temperature	1.11	4	0.28	2.72	0.12
Residual	0.71	7	0.10		
Total	1.82	11			

Table A1.26 Feeding protocol for KGW juveniles during temperature trial (20 and 25°C).

Days post hatch (dph)	20°C		25°C	
	Mean wet weight (g)	% bw/day	Mean wet weight (g)	% bw/day
222	1.9	5	2.17	5.7
229		3.8		4.4
236	2.75	5	3.42	5.75
250	3.59	5	5.38	4.5
265	4.31	5	7.04	5.75
278	5.34	3.8	9.72	4.4
292	6.88	3.8	11.45	4.4
307	7.95	3.8	14.89	4.4
320	10.14	3.8	18.93	3.35
334	12.73	3.8	23.58	4.4
348	16.13	3.8	29.72	4.4
362	19.18	3.8	32.32	4.2
376	22.17	3.8	36.81	4.2
390	23.83	2.1	40.65	3.15
404	27.24	2.1	44.64	3.15
418	31.8	3	49.73	3.25
432	35.02	3	55.79	3.25
449	40.58	3	61.69	3.25
464	44.33	3.4	67.86	3.5
477	47.65	3.4	73.45	3.5
491	46.94	3.4	75.30	3.5
505	57.07	2.43	83	2.31
520	52.96	2.43	82.24	2.31
533	63.81	2.43	82.96	2.31

Table A1.27 Feeding protocol for KGW juveniles in temperature treatments (20°C, 23°C and 26°C).

DPH	20°C		23°C		26°C	
	Mean wet weight (g)	Feed % bw/day	Mean wet weight (g)	Feed % bw/day	Mean wet weight (g)	Feed % bw/day
146	1.43		1.43		1.43	
161	1.92	4.19	2.18	5.28	2.45	3.5
175	2.60	5.37	3.23	5.99	2.99	3.5
189	2.70	5.56	3.60	3.37	3.95	3.5
203	3.58	6.65	5.46	5.91	4.85	3.5
218	4.45	5.01	6.86	3.95	6.95	5.41
231	5.26	4.75	7.77	4.78	8.53	3.47
251	8.80	5.00	11.31	4.18	10.57	5.15
265	10.17	4.01	11.66	3.37	14.24	3.47
280	13.70	4.30	14.80	4.07	18.53	6.00
294	15.95	3.35	19.02	3.50	22.47	4.49

Table A1.28 Two-sample t-test at 5% significance level to test the difference in final lengths (Standard length (mm)) of KGW juveniles grown at 20°C and 25°C.

t statistic	3.84
d.f.	100
P value	<0.01

Table A1.29 Analysis of variance at 5% significance level to test the difference in the mean weights (g) of juveniles grown out at different temperatures (20, 23 and 26°C).

Source	Sum of squares	DF	Mean Square	F-ratio	P
Temperature (A) Tank (B)	17790.70	2	8895.33	3.65	0.16
A*B	7308.59	3	2436.20		
Residual	157624	374	421.45		
Total	182723	379			

Appendix 2

Coastal Finfish Hatchery Manual: King George Whiting *(Sillaginodes punctata)*

Jane Ham and Wayne Hutchinson

This manual was produced as part of a three year (May 1997 - June 2000) Fisheries Research and Development Corporation (FRDC) funded research project 'Spawning and Larval rearing of King George whiting *(Sillaginodes punctata)* relevant to aquaculture and fisheries biology.' This project complimented investigations on the aquaculture of KGW supported through the "Farmed Seafood Initiative" of the South Australian Government.

This manual presents protocols developed and incorporates preliminary results of research trials conducted to date. It aims to cover the fundamental aspects of hatchery production for marine finfish in general using KGW as the focus species.

The manual is intended to be of use to people with an interest in marine finfish hatchery operations. It covers basic aquaculture principles as well as dealing with some topics in depth.

Coastal Finfish Hatchery Manual: King George Whiting *(Sillaginodes punctata)*

Jane Ham and Wayne Hutchinson

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Finally we wish to thank FRDC and the South Australian Government for their financial support, without which this manual would not have been possible.

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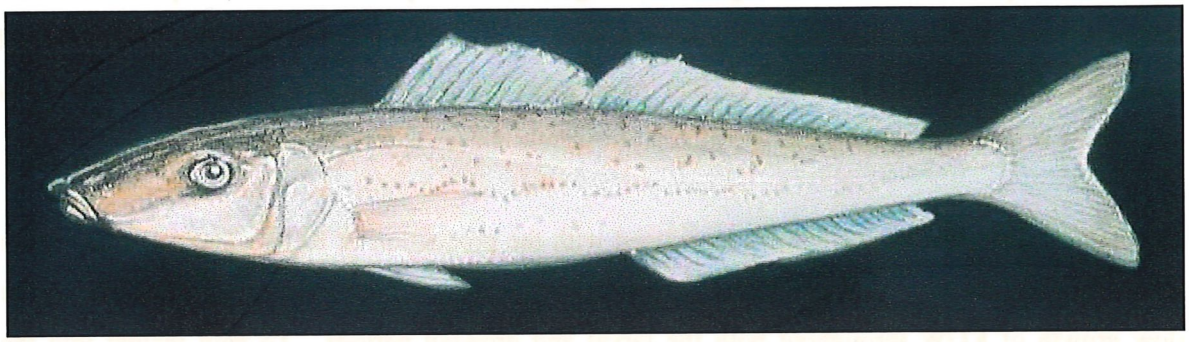


Figure 1 –1 Adult KGW

King George whiting (KGW) *Sillaginodes punctata* (figure 1-1), is a popular marine finfish highly prized in southern Australia by fishers and consumers.

Its delicate flavour, white flesh and fine texture, which keeps well after freezing, ensures its popularity and demand in the food industry. In South Australia, KGW is the most valuable species, with fillets fetching up to $40 per kg and whole fish up to $18 per kg (1999 prices).

Although commercial fisheries for this species also exist in Victoria and Western Australia, the fishery in South Australia is the most important (Kailola *et al* 1993). In 1998, KGW was the second most valuable marine finfish fishery in South Australia, behind Pilchards (Southern Fisheries, 1999). Consequently, this is one of the most heavily targeted species in South Australia, which has caused concern about the sustainability of the fishery.

The only way to meet current consumer demand without increasing pressure on wild stocks is production of this species through aquaculture. To date, limited projects and published information focusing on the development of methodologies for KGW culture exists.

Due to considerable interest in the potential for aquaculture of KGW for both commercial markets and re-stocking programs, a three-year (May 1997–June 2000) Fisheries Research and Development Corporation (FRDC) research project 'Spawning and larval rearing of King George whiting *(Sillaginodes punctata)* relevant to aquaculture and fisheries biology', was undertaken by The South Australian Research and Development Institute (SARDI) at The South Australian Aquatic Science Centre (SAASC). This was supplemented by a complimentary "Farmed Seafood Initiative" project through the South Australian Government.

Environmental parameters were assessed to develop commercially viable larval rearing and feeding protocols to optimise survival throughout all stages of hatchery culture. Other aspects of KGW culture investigated included achieving captive spawning of high quality eggs during the natural season and out of season. The research also investigated the growth rates and food conversion rates that can be achieved at different temperatures throughout the grow-out period of juveniles.

This study highlights the challengers associated with the production of KGW. The main issues are achieving a regular supply of good quality eggs from broodstock, poor hatchery production and the slow growth of KGW throughout both the larval and growout periods. To gain insight into the economic viability of growout of KGW in South Australia, 1g fingerlings have been stocked in a saline ponds at Port Augusta, in tanks using recirculating ground water at Posaqua, Tickera, and at the Bedford Saline Ground Water Interception Scheme at Cooke Plains, run by the Coorong District Council (figure 1-2). If these results prove promising then further research is recommended to address the major issues identified.

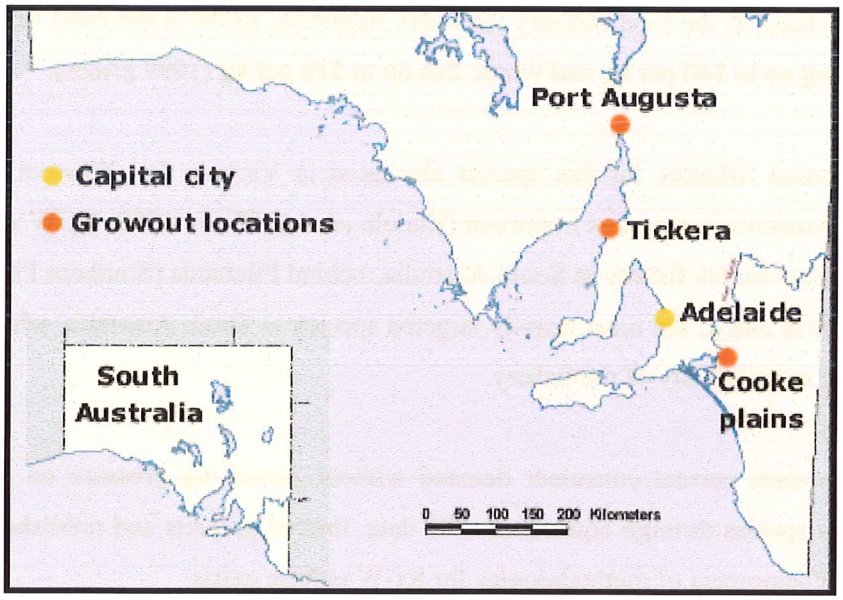


Figure 1 - 2 Map of the coastline of South Australia highlighting growout locations (Port Augusta, Tickera and Cooke Plains). State of South Australia displayed in the insert on the left.

The protocols presented in this manual have been developed throughout the FRDC research project. They are preliminary and incorporate the results of the research trials to date. Interactive compact discs of the 'Coastal Finfish Hatchery Manual: King George Whiting' are also available. Please contact SARDI Aquatic Sciences' library to obtain a copy, at PO Box 120, Henley Beach, SA, 5022. Phone: (08) 8200 2423. Fax: (08) 8200 2481. E-mail: bennett.suzanne@saugov.sa.gov.au

Chapter 2: Background

2.1 Biology

KGW, the largest Australian whiting species from the family *Sillaginidae*, is endemic to Australia (Figure 2-1). Its distribution stretches across southern temperate waters from Port Jackson (Sydney) in New South Wales to Jurien Bay in Western Australia, including northern Tasmania (Kailola *et al* 1993).

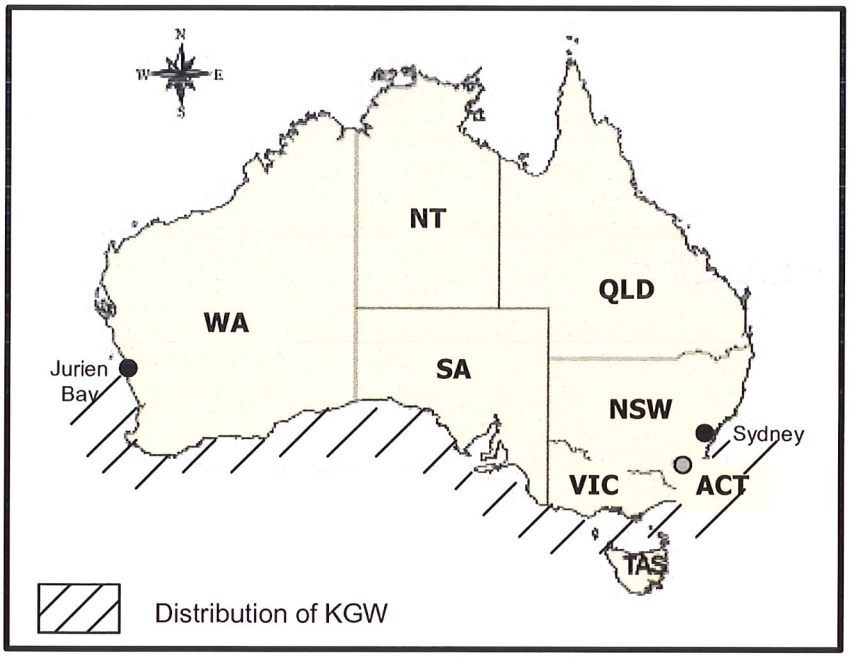


Figure 2 - 1 Distribution of KGW

Whiting have an elongated body with a pointed snout. KGW are easily distinguished from other whiting by their tiny scales in the lateral line and dark spots on the sides. They have two dorsal fins, often slightly separated, the first with 11–13 spines and the second with one spine and 25–27 soft rays (Yearsley *et al* 1999). The caudal fin is forked (Figure 2-2).

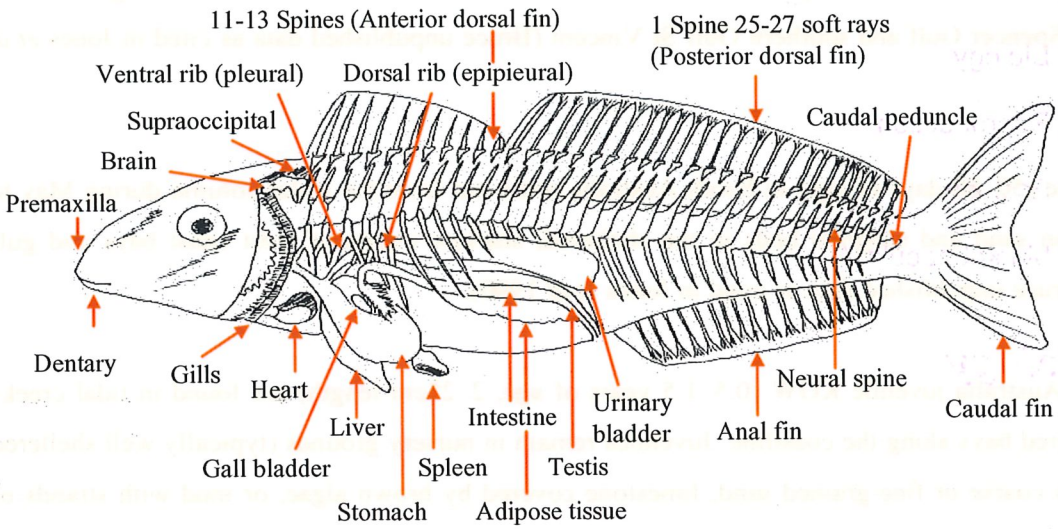


Figure 2 – 2 Diagram of adult KGW indicating skeletal structure and location of major organs (modified from Caillet *et al* 1986)

Most KGW taken from the fishery are in the size range of 30–40cm, however, fish longer than 50cm are taken from some regions. Most fish are less than 400g, although occasionally they may exceed 1kg (Fowler and McGarvey 2000).

2.2 Reproduction

In South Australia mature KGW spawn between February and July, with the peak in April (Scott 1954; Bruce 1989; Cockrum and Jones 1992 as cited in Fowler and McGarvey 1997). In Victoria they spawn from mid-April to mid-July peaking from mid-May to early July (Jenkins and May 1994 as cited in Fowler and McGarvey 1997); and in Western Australia starting in June and continuing until August (Hyndes *et al* submitted as cited in Fowler and McGarvey 1997).

Length at first maturity is 27cm for male KGW and 32cm for females (Cockrum and Jones 1992). Most females are reproductively mature at the end of their 3rd year and by the 4th year most are reproductively active (Fowler 1998).

2.3 Ecology

Adults inhabit exposed waters along coastal beaches and reef areas, sometimes to depths as great as 200m (Kailola *et al* 1993).

In South Australia spawning occurs in deeper waters south of the Gulf of St. Vincent and Spencer Gulf. Eggs and larvae are transported via ocean currents and have been located in Investigator Strait, southern Spencer Gulf and southern Gulf St Vincent (Bruce unpublished data as cited in Jones *et al* 1990).

Post larvae (50–80 days of age) in South Australia settle out from the water column during May to October on sand and seagrass beds in the sheltered, shallow waters of west coast bays and gulf waters (Bruce unpublished data as cited in Jones *et al* 1990).

In South Australia juvenile KGW (0.5–1.5 years of age, 2–25cm length) are found in tidal creeks and sheltered bays along the coastline. Juveniles remain in nursery grounds (typically well sheltered areas with coarse or fine-grained sand, limestone covered by brown algae, or mud with strands of the seagrass *Zostera* as a substrate) for up to 12 months (10–15cm in length) then move to slightly deeper and more exposed waters adjacent to their nursery ground. Fish (20–25cm in length) move out further on to seagrass-covered sand areas and remain there for about another 12 months until they reach about 36–37cm at 3–4 years of age (Jones *et al* 1990).

2.4 Market popularity

KGW have an established market in southern Australia. Consumers consider KGW as a fish of high quality and great taste, which ensures its demand. Due to its popularity it has become one of the most valuable fish species in temperate Australia (up to $42 per kg fillets, 2000 prices).

A range of sizes of KGW is sold at retail markets. Whole KGW are classified into categories (table 2 - 1).

Table 2 -1 Weight ranges (g) of whole fish and average recovery rates (percentage of meat recovered from whole KGW after filleting) of respective size classes of KGW sold at retail markets

Size class	Weight range (g)	Average recovery rate (%)
Small	170-190	57
Medium	190-220	57
Medium/large	220-230	57
Large	230-250	62
Extra large	300-340	62

KGW has captured the interest of fish farmers because of its high value and the opportunities in both the established and potential markets. Currently, its supply is seasonal with limited amounts available over summer. Its unpredictable supply often causes the price to fluctuate dramatically. The

combined result is that many restaurants no longer use KGW. Food industry responses suggest that demand would increase if a consistent supply of good quality KGW could be delivered year round.

A consistent supply, allowing restaurants to incorporate KGW into their regular menus, could extend the current market, particularly in New South Wales and Victoria. Currently the amount sold in South Australia is approximately equal to that in Victoria with little sold in NSW (Figure 2-3). The higher populations of these States suggest that with a reliable supply their markets could be expanded significantly.

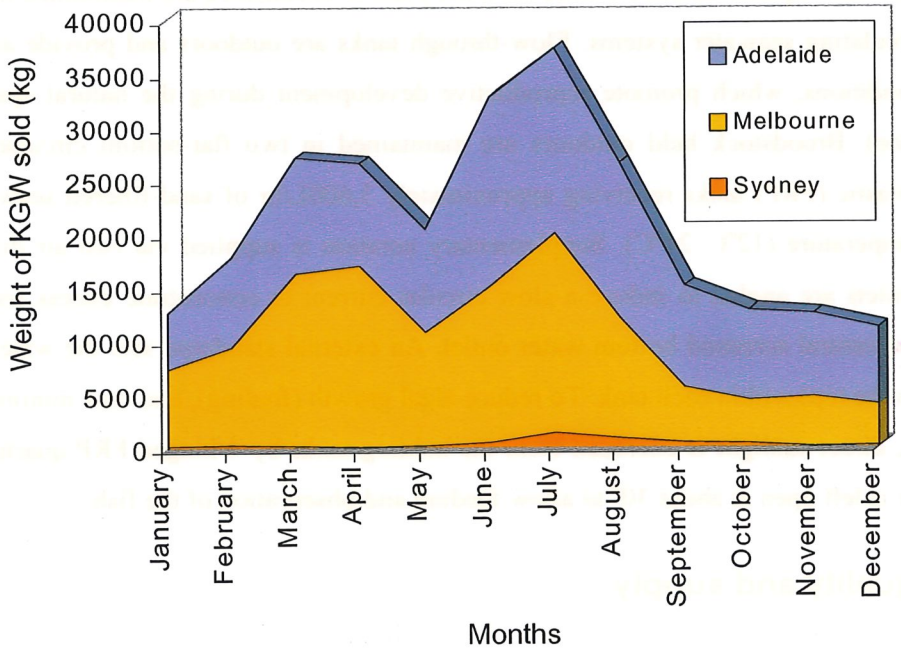


Figure 2 –3 Volume (kg) of KGW sold in Safcol fish market (Adelaide), Sydney and Melbourne fish markets over a one-year period (from www.sydneyfishmarket.com.au; www.chsmith.com.au/fish-species/melbourne.html; Safcol fish market records).

Chapter 3: Broodstock systems

3.1 Broodstock facilities

3.1.1 Tanks

At the South Australian Aquatic Science Centre (SAASC) KGW broodstock are maintained in flow-through and recirculating seawater systems. Flow-through tanks are outdoors and provide ambient environmental conditions, which promote reproductive development during the natural spawning season (April–June). Broodstock held outdoors are maintained in two flat-bottom circular 40kL fibre-reinforced plastic (FRP) tanks receiving approximately 3,600L/hr of sand-filtered seawater at ambient water temperature (12°C–24°C). Supplementary aeration is supplied via two air diffusers per tank. Water inlets are angled to induce a slow circular current to concentrate excess feed and faeces towards the central screened bottom water outlet. An external standpipe sets the water level (about 30cm from the top) within each tank. To reduce algal growth (fouling), and thus minimise the cleaning required, direct sunlight is excluded from the holding tanks by 4 hinged FRP quarter tank covers. One cover is left open at about 30° to allow feeding and observation of the fish.

3.1.2 Water quality and supply

Optimum water quality should be maintained in holding tanks at all times to maximise the reproductive performance of broodstock. High water-quality conditions are achieved through system design and operation, with attention to:

- water flow rates required to achieve the desired water exchange in broodstock holding systems,
- not overfeeding broodstock and efficient removal of uneaten food and wastes from tanks and filtration systems, and
- minimising stocking densities to achieve the desired outcomes (avoid overstocking).

At SAASC, seawater is sourced from Gulf St Vincent via 325mm intake pipes 1400 and 1440m offshore. Seawater flows by positive head into a 14m deep concrete caisson. Raw seawater is first pumped through a coarse brush screen, which removes large particles such as pieces of weed,

shellfish and fouling organisms. Coarse filtered water then flows to a central distribution manifold within each of three 180kL primary settlement tanks. The central manifold allows water to fan passively into the tank, creating minimal agitation while tank retention time allows fine suspended particles to settle. Seawater is then skimmed from the surface layer and pumped through two pressure sand filters (Chadson Engineering Western Australia, 1.8m^2, 12.6L/sec/m^2) which remove particles to 20–30 µm prior to transfer into 2 x 250kL internal-membrane-coated concrete storage tanks. These tanks provide a 12-hour water storage capacity and water supply to multiple pumps in a plant room, from where filtered seawater is distributed to a number of locations. The maximum capacity of the seawater supply system is 2 kL/minute.

3.1.3 Water flow and stocking density

It is recommended that stocking densities of broodstock be maintained between 2–5kg/kL. These low stocking densities encourage good reproductive performance and make it easier to maintain high water quality in flow-through tanks or relatively simple recirculation systems. At these stocking densities, flow-through tanks for holding KGW broodstock should receive sufficient water exchange to maintain water quality. Water flow rates should also be sufficient to remove eggs spawned within a 12-hour period. Morning collection of evening spawn can be achieved if the water flow rate in a flow-through system is a minimum exchange of four times the tank volume per day. Within recirculation systems, higher flow rates are generally used to achieve water exchange in the order of six times the tank volume per day (25% tank volume exchanged per hour).

3.1.4 Salinity

Juvenile KGW can be found in the upper Spencer Gulf region of South Australia where they are exposed to elevated salinities of up to 48 parts per thousand (ppt). Adults are generally found in deeper more oceanic waters with salinities of 35–38ppt, which are thus recommended for holding broodstock.

3.1.5 Water temperature

Outdoor broodstock holding tanks at SAASC are maintained at ambient water temperature which ranges from 12°C – 24°C. This thermal range, which has been found to induce natural spawning, is used as the basis for the environmental manipulations to which broodstock held in controlled systems are exposed.

3.1.6 Dissolved oxygen

Dissolved oxygen (DO_2) levels should be maintained as close as possible to 100% saturation for the prevailing conditions of salinity and water temperature (Section 11.8: Dissolved oxygen table). Levels of DO_2 should be checked periodically when the water is warm, as oxygen-carrying capacity declines and consumption by fish increases, particularly following heavy feeding.

The level of DO_2 can be easily monitored using readily available portable electronic meters. Problems with DO_2 may be indicated by fish swimming at the surface, and/or congregating at water inlets, flared gills, rapid opercular movements and lethargic swimming activity.

Increased aeration, the addition of spray bars to water inlets, extra air diffusers, increased water flow, removal of uneaten feed and faeces, and the reduction of feeding intensity, can be used to improve DO_2 levels in holding systems.

3.1.7 Nutrients

Elevated levels (>110% saturation) of dissolved nitrogen gas (N_2) can be lethal to fish and effects can be observed as low as 102% saturation (Tucker 1998). Supersaturation of N_2 results in bubble formation within blood vessels and tissue damage (gas bubble disease) which is often seen as 'pop eye' in broodstock, and over-inflation of swim bladders in larval fish.

The most common causes of supersaturation are leaking plumbing connections on the suction side of pumps, surge in pipes (air entering under pressure), rapid cooling of heated water and sudden increases in barometric pressure (Tucker 1998). If supersaturation is suspected in systems, efficient packed column or vacuum degassers should be installed.

An indication of the levels of dissolved N_2 can be measured using a total dissolved gas meter, which also measures dissolved carbon dioxide. Total gas meters are not currently widely available, however.

3.1.8 Ammonia

Refer to section 3.2.3 Biological Filtration: Recirculation Systems.

3.2 Recirculating seawater treatment systems

A 40kL capacity (4 x 10kL tanks) recirculating seawater treatment system (RSTS) was installed at SAASC to maintain high-quality seawater for KGW broodstock. This system was used to condition broodstock to spawn outside the natural season (October–November) through manipulation of key environmental parameters (that is water temperature and photoperiod). Out of season egg production can accelerate research progress and allow commercial hatcheries to provide stock to on-growers at times which best suit production cycles.

There are many types of recirculating systems but the fundamental principles and key components are similar. Described here are the components and design of the system at SAASC (for more detailed technical information consult Hart and Sullivan 1993).

To facilitate temperature control components of the RSTS are housed in a 10m x 8m shed (Figure 3 - 1) of Bondor type metal clad insulated panel (100mm thick). The system (Figure 3-2) holds about 50kL of seawater with 25kL/hr pumped through the sand filter after which total flow is side streamed to a biological filter/foam fractionator (15kL/hr) and the remaining 10 kL/hr returned to the tanks (2.5kL/hr per tank, 25% exchange/hr). A summary of water flow-through the system is provided in Figure 3 - 3.

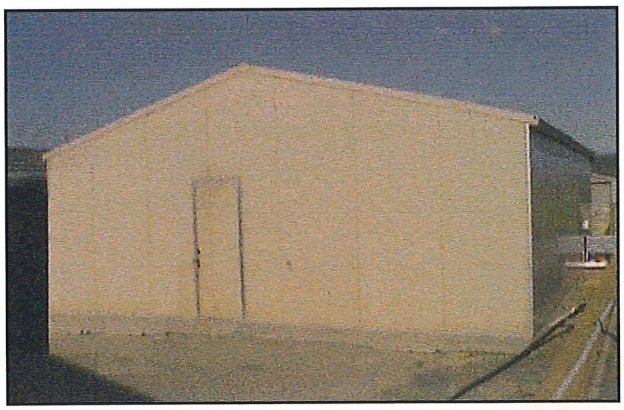


Figure 3 - 1 Broodstock shed at SAASC

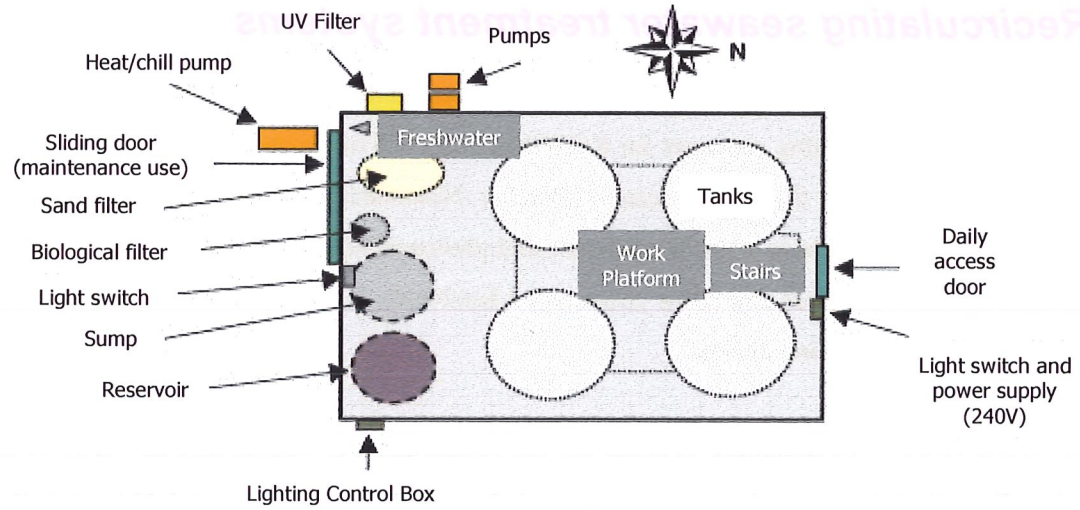


Figure 3 - 2 Layout of broodstock shed at SAASC

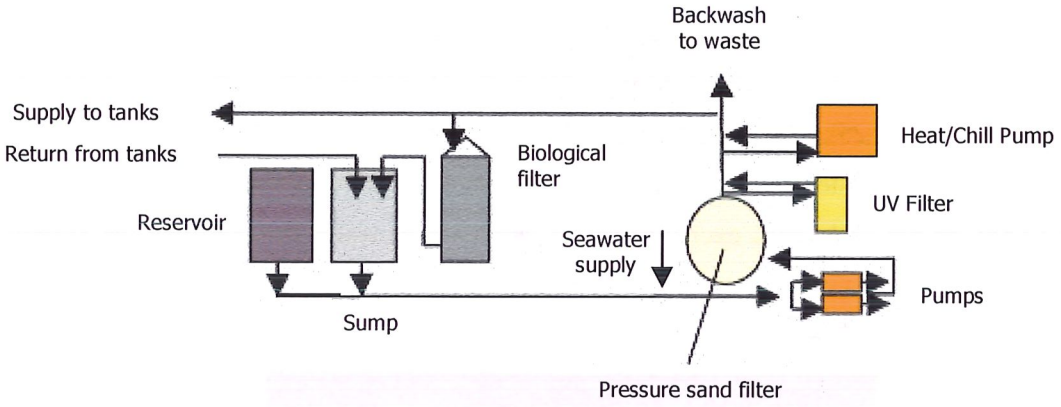


Figure 3 - 3 Overview of water flow in recirculating system at SAASC

3.2.1 Mechanical filtration

Mechanical filtration in the system removes suspended solids such as faeces and uneaten food. This is done in the first stage of water treatment in the RSTS to prevent this material accumulating in other components of the system.

There are many methods of mechanical filtration such as: surface filtration (stationary screens, rotating drum and disc screens); diatomaceous earth (DE) filters; depth filters such as gravity sand filters and pressure sand filters; and cartridge filters (Hutchinson and Forteath 1993 in Hart and O'Sullivan 1993).

All seawater entering the broodstock shed has been pre-filtered to a nominal 30µm with pressure sand filters, which treat all intake seawater at SAASC. Within the system a 50kL/hr capacity

pressure sand filter (Chadson Engineering Western Australia, 1.5m^2, 55kL/hr) provides continuous mechanical filtration of all water returning from broodstock tanks (Figure 3-4).

Pressure sand filters are depth filters with water forced through the sand particles, trapping solid particles and allowing filtered water to exit through a slotted manifold in the base of the filter vessel. This system requires twice weekly back flushing to remove the build-up of solids. The use of pressure sand filters can be problematic if backwashing is not efficient or with high stocking densities of fish.

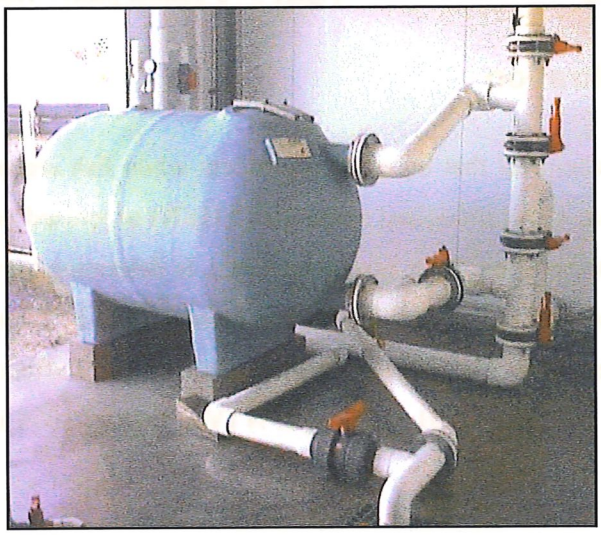


Figure 3 - 4 Pressure sand filter

3.2.2 Heat/chill pumps

A heat/chill pump (Carrier Model 30ZQC060) in the system controls water temperature (Figure 3-5 and 3-6). About 5 kL/hr of the water directly after the sand filter is side-streamed to the heat/chill pump and returns to the main distribution line before it is split to the biological filter and tank supply lines. The heat/chill pump is outside the shed, to reduce noise and provide ventilation required for heat/cold transfer from the system. It has a thermostat control, which is manually adjusted each week following a predetermined water temperature regime timed to advance or retard spawning.

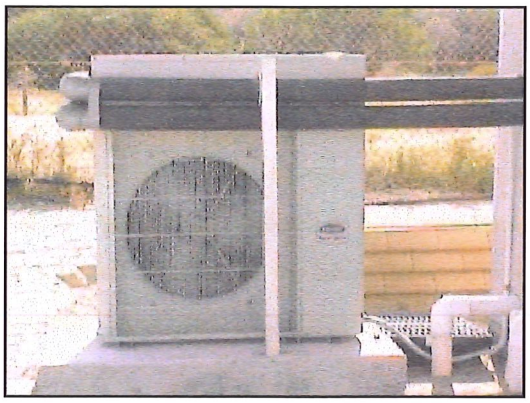


Figure 3 - 5 Heat/chill pump

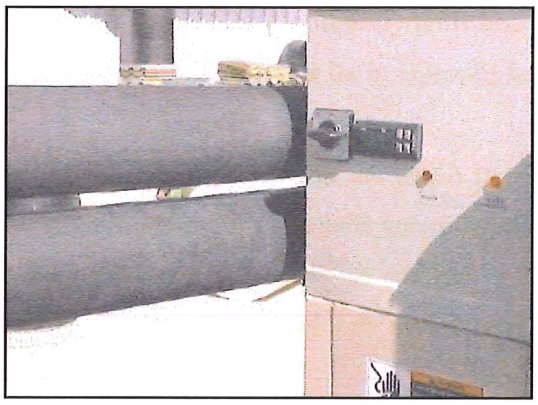


Figure 3 - 6 Control panel – Heat/chill pump

3.2.3 Biological filtration

Ammonia is the major chemical that becomes a problem when fish are maintained in recirculated water. It exists in water as either unionised ammonia (NH_3-N) or ionised ammonium (NH_4^+-N). The equilibrium, which exists between these two compounds, is determined primarily by pH, with high pH increasing the proportion existing as NH_3-N (Van Gorder 1994 in Timmons and Losordo 1994). While NH_4^+-N is harmless to fish NH_3-N is extremely toxic and will affect fish at levels <0.02mg/L (Rogers and Klemetson 1985) and 0.01–0.05 mg/L N (Van Gorder 1994 in Timmons and Losordo 1994).

Ammonia levels within the RSTS are reduced by biological filters designed to promote oxidation of this compound to nitrate, which is relatively harmless to fish at the levels (<100mg/L) in these systems (Forteath 1990). Oxidation of ammonia to nitrite is accomplished by aerobic bacteria, mainly *Nitrosomonas sp.* Nitrite is then oxidised to nitrate by another group of bacteria, *Nitrobacter sp.* Biological filters are constructed to provide high amounts of surface area for colonisation by these beneficial bacterial species while allowing sufficient water flow to provide the oxygen these aerobic organisms require.

Biological filters should be designed to provide a large surface area sufficient to treat the predicted metabolic waste load from the biomass of fish in the system. A range of inert plastic biological filter balls (bioballs) are available (Figure 3-7) for construction of biological filters. Each design has a characteristic high surface area/unit volume and void space to allow water flow and gas exchange. High oxygen levels need to be maintained in biological filters as nitrifying bacteria are highly aerobic and optimal performance will not be achieved if DO_2 is reduced.

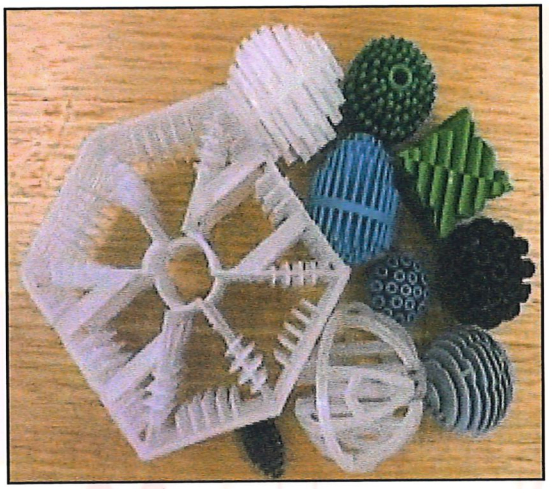


Figure 3 - 7 A sample of biological filter balls that are available

Biological filters should be set up several weeks before stocking fish to allow populations of nitrifying bacteria to build up to levels which can treat the wastes from fish. Commercial preparations of selected nitrifying bacteria are available from aquarium shops and aquaculture suppliers. At SAASC, these starter cultures have been used in combination with a daily addition of 50mg/L of ammonium chloride (NH_4Cl) and 75mg/L of sodium nitrite ($NaNO_2$), to promote growth of both types of nitrifying bacteria until fish can be progressively stocked into the system and start producing ammonia.

The biological filter in the RSTS for out-of-season spawning of KGW contains 0.75m^3 of bioballs (144m^2/m^3, Otto Plastics, Figure 3-7) in a 0.8m diameter x 2.4m high FRP tank. The design provides a submerged bed of bioballs with a counter current of water (ie. top inlet and bottom outlet) flowing against aeration up through the bed provided by 6 x 150mm diameter diffusers at the base of the tank (Figure 3-9). As bioballs generally float, the bed is held submerged by a 20mm plastic mesh screen secured 0.8m from the top of the tank or 0.3m below the operating water level.

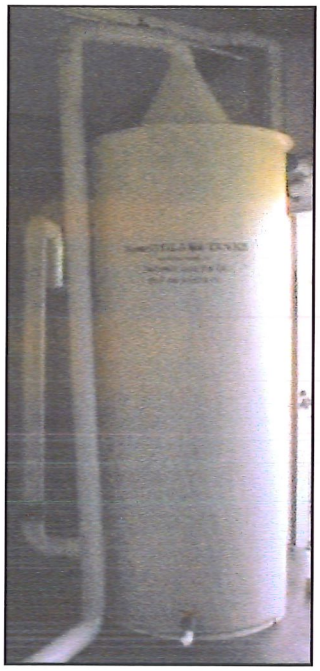


Figure 3 - 8 Biological filter

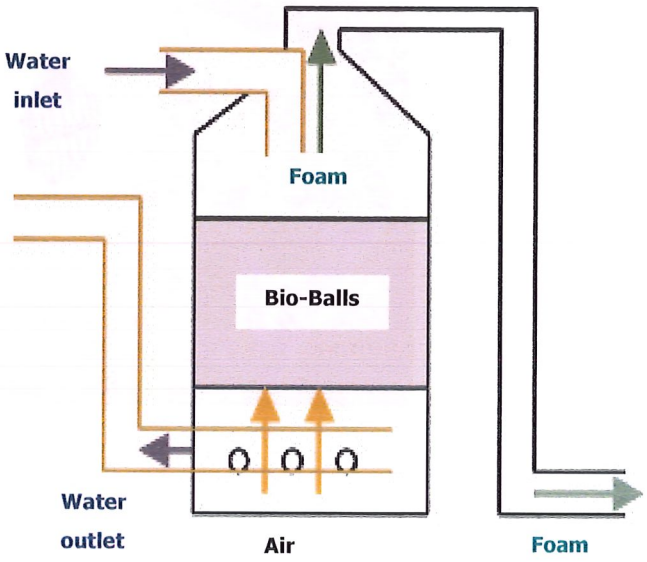


Figure 3 - 9 Cross section diagram of biological filter

3.2.4 Foam fractionator

Foam fractionators concentrate and facilitate the removal of surface active components of dissolved organic carbon (DOC) which accumulate in recirculating aquaculture systems and eventually turn the water a yellowish brown. Surface active molecules dissolved in water have both hydrophobic (repelled by water) and hydrophilic (attracted to water) ends. This causes them to congregate at bubble surfaces with hydrophobic ends within the bubble while the hydrophilic end is immersed in water. This mechanisms allow surface active DOC (ie. some proteins, amino acids, fats and carbohydrates) to be concentrated on bubble surfaces and stripped from water if the resultant foam can be separated by a foam fractionation device.

Foam fractionation at SAASC is incorporated into the top of the biological filter removing foam containing DOC. Although this is not an efficient purpose-built foam fractionation device, it takes advantage of the foam generated through aeration of the biofilter bed. Air bubbles moving up through the column attract DOC on their surfaces, which accumulates as thick foam at the surface of the tank. When enough has built up, it is forced through the conical top and down the discharge tube, where coagulated material or 'scum' is removed from the system.

3.2.5 Tanks

In the system, broodstock are maintained within 4 x 10kL capacity (2.7m diameter x 2.0m high) rotational moulded FRP tanks (Outback Tanks, Bundaberg, Queensland). Bottom drains and adjustable taps on inlet and outlet drains are fitted to each tank. Inlet pipes are angled to create a circular flow within tanks. Two air diffusers are operated in each tank. Nets over the top of tanks prevent fish jumping out if startled (Figure 3-10).

Each tank can connect a 500µm mesh egg collector bag fitted in a round tub (114L, Nally) during the spawning period. A tap on the external standpipe is used to divert water through the bottom tank outlet or through the egg collector via a tank outlet surface at the water surface. This allows water and wastes to be treated by the RSTS during the day and eggs to be skimmed from the surface for periods, which suit the identified spawning time, usually after dusk. Returning the tap to normal operating position stops flow to the egg collector and allows eggs to be settled and removed from the mesh bag.

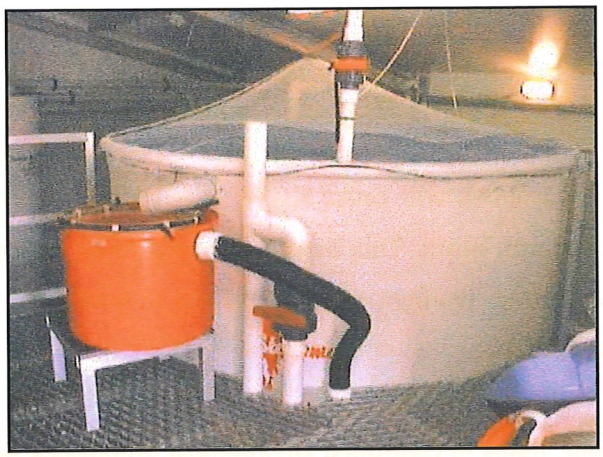


Figure 3 - 10 Broodstock tanks with nets and egg collector attached

3.2.6 Sump and reservoir

The sump receives water from tanks and supplies water to the pump. It has a total volume of 4kL and must remain full at all times. Additional water (4kL) is stored in the reservoir (pre-filled to adjust to room temperature) and is introduced to the system during a water exchange, minimising temperature fluctuations during weekly exchanges (Figure 3-11). Both the sump and reservoir are polyethylene tanks (1.7m diameter x 1.85m high, Master Tanks, 230 Richmond Rd, Marleston, SA).

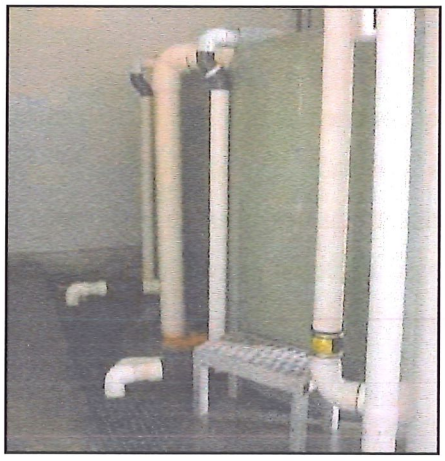


Figure 3 - 11 Sump and reservoir

3.2.7 Pumps

Two 3-phase (415 volt/50 Hertz) Grundfos centrifugal pumps 380–420L/min (22.8–25.2kL/hr) operate the system with one run on alternate weeks and both used during filter backwash. Pumps are outside the building to reduce noise and to remove an unwanted minor heat source from inside the insulated building.

3.2.8 Lighting

Sudden bright lights or loud noises will cause KGW to swim into walls of tanks or jump, often resulting in harm and sometimes death. This behaviour needs to be anticipated, with care taken to enter broodstock facilities during 'daylight' periods and not to startle fish with sudden loud noises (opening or slamming doors etc).

To minimise this behaviour, lights in controlled environment broodstock holding facilities are fitted with adjustable timers to allow the photoperiod to be manipulated and faders to slowly ramp illumination up and down. Two light circuits are installed with separate timer controls. One circuit powers lower intensity wall mounted incandescent lights (100W) controlled by a timer connected to a fader (Intelux Intelligent Lighting Control, Keltsrom Pty Ltd, Victoria). The wall lights turn on first and off last to provide a simulated dawn and dusk over a 60-minute period. The other circuit powers higher intensity fluorescent lighting mounted above tanks. These lights are controlled by a standard timer and turn on after and off before the wall mounted fading lights to simulate daylight. Weekly day length adjustments are made to timer settings to advance or retard spawning (see section 6.2: Environmental manipulation for information on manipulating the photoperiod). These are done at the same time as adjustments to the heat/chill unit (water temperature).

3.2.9 Water quality

See Broodstock Facilities: Section 3.1.2

3.2.10 Ultraviolet filter

Ultraviolet (UV) filters are used to disinfect water through exposure of micro-organisms to destructive or bactericidal light wavelength of 254 nm (Cailleres 1999), which penetrate cell membranes to disrupt DNA molecules and thus prevent cell division.

Generally, these filters house UV tubes which illuminate water passing in close proximity. UV filters can be incorporated into systems where a percentage of water is by-passed through the filter so that the total volume in the system is treated 2–4 times per day. UV filters will then provide a level of disinfection which reduces, but does not eliminate, the level of micro-organisms within the system.

Professional advice is needed to determine what size (ie. flow capacity and power output) and configuration of UV filter is suitable for particular systems.

Chapter 4: Broodstock Husbandry

4.1 Collection

For commercial production and accelerated research, it is desirable to have controlled production of eggs available from spawning broodstock at regular intervals throughout the year.

4.1.1 Hatchery reared broodstock

Cultured fish can be maintained in captivity until they reach maturity and are used as broodstock. Studies are under way to look at the time to maturity, egg production numbers and quality of hatchery-reared broodstock. As these fish are fully domesticated, the spawning fraction may be higher than wild-caught broodstock, producing larger numbers of high quality eggs.

4.1.2 Collection of broodstock from the wild

Note: In South Australia, the holding of fish in tanks and capture of wild fish for the purposes of aquaculture requires licenses (see Section 11.16).

KGW adults are found in coastal marine areas, usually in depths less than 50m (reported up to 200m) over sand or seagrass.

SARDI captured KGW for broodstock by line, using barbless hooks. To date, incision wounds to the mouth region have healed relatively quickly if not too severe. Fish hooked in the gills digestive tract or with severe damage from capture were euthanased.

To maximise survival during the collection of fish, it is important to keep the following points in mind:

- minimise handling that may cause removal of scales and mucous (wearing latex gloves while handling fish can assist with this),
- avoid overcrowding of fish in transporters (up to 40kg/m^3), and
- maintain optimum environmental conditions in fish holding tanks (temperature and salinity should be the same as at the capture site and dissolved oxygen should be maintained between 8–12mg/L).

4.1.3 Transport

Holding tanks

Once hooked, fish are placed directly into 200L cylindrical holding tanks (Figure 4-1) secured on the deck of the boat. Holding tanks have a 12-volt bilge pump attached to the extended end of a 20mm diameter inlet hose reaching over the side of the boat and below the water surface allowing water to be pumped up from the sea. The other end of the inlet hose reaches to the bottom of the holding tank, creating a constant water exchange in the tank. A 40mm diameter overflow hose is attached above the inlet hose, removing water from the top of the tub. Cables from the bilge pump are attached to a 12-volt boat battery or a 12-volt power supply point. When the boat is in motion, the bilge pump is turned off and placed into the holding tank to avoid siphoning.

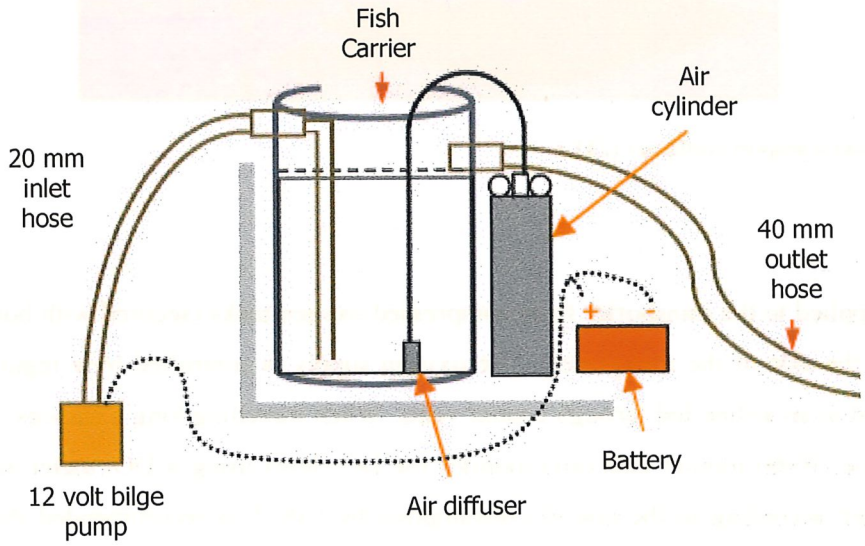


Figure 4 - 1 Schematic diagram of on-board tanks holding and transport of KGW broodstock

Air supply

When bilge pumps are not in use (ie. travelling between fishing locations), it is necessary to provide aeration to holding tanks. Air is supplied from industrial air cylinders by a regulator and diffuser (Figure 4-1). Gas cylinders are secured against the side of the boat.

Road transport container

To move live fish, road transport tanks (400L – 2kL) are used (Figure 4-2). Transporters are secured on a trailer fitted with an air-assisted braking system. Prior to stocking, the transporter is partially filled with seawater of similar water to the fish collection site. Once fish are stocked, the transporter is topped up with seawater leaving a 10cm air space and the lid is fastened to minimise water loss during travel.

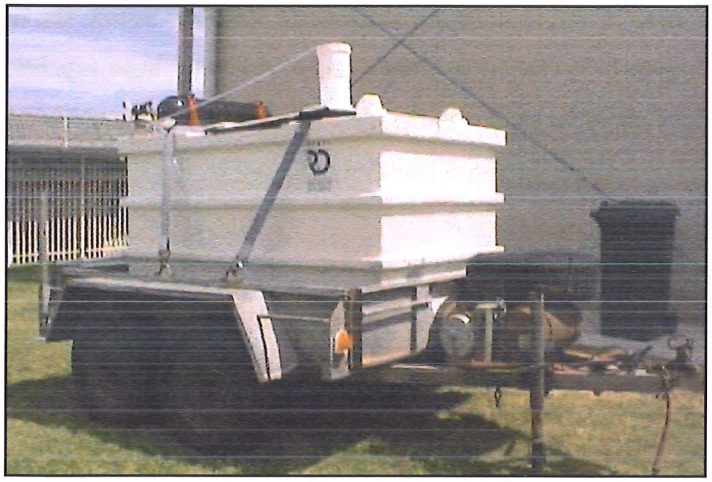


Figure 4 - 2 Road transport container (2kL)

Oxygen

Oxygen is supplied to the transporter from compressed oxygen tanks (secured with holding brackets on top or on the side of the transporter). The oxygen supply is controlled by a regulator and flow meter attached to an airline fed through central vents. When travelling long distances, it is crucial to periodically (ie. 30-60 minute intervals) monitor the DO_2 level using a DO_2 meter and adjusts the flow of oxygen according to the rate of consumption by fish. It is recommended that the oxygen level be kept at 100% saturation level, which is generally between 8–12mg/L.

Venting

Accumulation of carbon dioxide can be lethal to fish during long trips. This is of more concern at high densities associated with delivery of fingerlings for on-growing. It is essential that vents are

fitted to fish transporters and a small amount of head space above the water level (ie. 50–100mm) is left to allow exchange of gases. Water turbulence due to movement and oxygenation is usually sufficient to promote gas exchange at the water surface and flushing of CO_2 through vents.

Anaesthetics

A small amount of anaesthetic (Section 4.3) can be added when moving fish to reduce stress. SARDI has not found this necessary with KGW, which settle in dark coloured tanks or when lids are secured. Anaesthetics are routinely used to sedate fish during transfer from on-board tanks to holding tanks at the final destination. Generally, the anaesthetic dosage used is mild (ie. 10-50% normal dose), to induce sufficient disorientation to allow quick capture and reduce stress during the required handling procedures.

Nets/slings

Fish can be moved using a soft knotless net (0.5cm mesh) or by using slings (a rectangular polypipe frame that is flexible in the middle, covered in smooth vinyl with small holes cut through to allow water flow (Figure 4 - 5). Slings are preferable to move fish as nets can cause damage if fish are thrashing.

Stocking fish into tanks

Fish are moved from the transporter to the tanks on arrival using the methods described above. If conditions in the holding tank are different (ie. temperature and salinity) from those of water in the transporter, some acclimatisation of fish will be required. This can often best be achieved by running water at the destination into the transport tank and allowing this to overflow for 30–60 minutes before stocking fish.

4.2 Handling

When working with fish, it is important to minimise handling and impose as little stress as possible. Where possible, all capture and transfer procedures should be conducted on partially or fully anaesthetised fish using slings (Figure 4-5) rather than nets.

To pick up fish, cup one hand gently around the snout of the fish and use the other hand to support the back end of the fish, holding the fish ventral side up (Figure 4-3). This technique is particularly important during the spawning season as any pressure on the abdomen of the fish can cause spontaneous release of milt or eggs.

Figure 4 - 3 Correct handling procedure of an anaesthetised fish (one hand is cupped around the snout, the other supports the back of the fish)

4.2.1 Capturing fish in a tank

The following procedure is used at SAASC to capture fish in a tank (Figure 4-4). While relatively labour intensive, it imposes minimum stress.

Procedure for capturing fish

- Drain tank to a water depth of about 40cm. It is important to ensure adequate aeration as stressed fish can rapidly deplete DO_2 in small volumes of water.
- Place tank divider (made of polypipe frame and smooth vinyl with holes cut through it to allow water flow) in tank to 'corral' fish.
- Place one end of the divider flush against the tank and secure with sand bags (tough plastic bags half-filled with sand and with air removed).
- Move the other end of the divider around the tank until the fish are isolated in a reasonable amount of space, then secure with sand bags (Figure 4-4).
- Fill tub with water and anaesthetic solution (Section 4.3 for doses and additional information on anaesthetising fish).
- Use a sling to capture individual fish by pointing the ridged end of the sling into the water (Figure 4-5). Immerse about half of the sling under water and allow the fish to swim in head first. Close the sling once the fish is inside and hold it on a slight angle, head end up, so that the fish cannot swim out the other end.
- Place the sling in the anaesthetic tub and tip it head down to allow the fish to swim out head first.

- Once fish have been anaesthetised and handling procedures are completed, place fish in an aerated recovery tub (200L tub) with water flow if possible. Once full movement is restored, return fish to the tank.

Figure 4 - 4 Capturing fish in a tank using a tank divider

Figure 4 - 5 Using a sling to catch KGW

4.3 Anaesthetics

To reduce damage and stress to fish during handling, anaesthetics are commonly used to mildly sedate or fully anaesthetise them.

Many anaesthetics are available in fish culture, including benzocaine, clove oil, AQUI-S™, Tricaine methane sulphonate (MS-222), quianaldine sulphate, 2-Phenoxyethanol and metomidate. **Note: Do not use any chemicals mentioned in this manual without the approval of relevant state and federal authority. Please contact the National Registration Authority (NRA) on (02) 6272 5852 or access their website www.nra.gov.au for current permit status**. Many studies (Section 11.15)

have looked at the effectiveness and safety of anaesthetics to both the fish, the technician and the consumer. Ideally, an anaesthetic should be used that anaesthetises fish quickly, is easily administered, provides fast recovery, has a wide safety margin, is low cost, has a long stable shelf life and breaks down rapidly in the environment (Stoskopf, 1993). Benzocaine is widely used at SAASC and will be discussed in detail, as will clove oil, a cheaper and to some degree, safer alternative.

4.3.1 Procedure for anaesthetising fish

- Prepare anaesthetic and recovery tub (100–200L tubs are adequate for KGW). Fill to the desired volumes with the same water as in the tank. Aerate tub, as oxygen depletion can be a significant problem when anaesthetising fish by immersion (particularly when many fish are being passed through the same induction water, Stoskopf 1993).
- Place a stock solution of anaesthetic in the tub (refer to later sections for information on preparing stock solutions and dose rates). If the anaesthetic dose is correct, fish should become disorientated and 'roll over' in 3–4 minutes. Once fish turn ventral side up, they should be left for another minute to become fully anaesthetised (at this stage, gill and tail movements will slow).
- Monitor fish behaviour during anaesthesia as fish in poor health require much lower doses of anaesthesia and are easily overdosed.
- It is recommended that measuring, inspection or induction procedures be restricted to 2–3 minutes, after which fish should be returned to the recovery tub. Fish can be assisted in their recovery from anaesthetics by opening their mouths and gently forcing fresh seawater over their gills. Once the fish regains jaw tone, it should be left to recover without assistance in a safe environment (Stoskopf 1993). A general rule is that if fish take a long time to become anaesthetised, they will take a long time to revive.

4.3.2 Benzocaine

Benzocaine (P-aminobenzoate) **(contact NRA for current permit status)** is a colourless crystal or white odourless crystalline powder, which is virtually insoluble in water and is prepared in 95% denatured ethanol. (it has been noted that this can be irritating to some fish species although this has not been recorded in KGW). It is possible to obtain the salt of the drug, benzocaine hydrochloride, which is soluble in water. However, it is much more expensive. All stock solutions of benzocaine must be stored in opaque bottles or in dark conditions as the drug and its salt break down in sunlight.

Benzocaine has a relatively wide safety margin in immersion time. However, if fish are left in anaesthetic for up to 3 times the recommended time, doses can become lethal. For KGW immersion times of no longer than 5 minutes at the recommended dose is suggested. It is also important to note that while the anaesthetic is less effective in warmer waters thereby often requiring the use of higher doses, the chemical is more toxic at higher temperatures which can result in overdoses (Stoskopf 1993). Previous studies indicate that benzocaine can be used without the long withdrawal periods (ie. the time between treatment and harvest of the product) that can be necessary with other chemicals such as MS-222 (Gilderhus 1990, Gilderhus *et al* 1991).

Preparation of Benzocaine

An effective dose of benzocaine for KGW adults has been found to be 60ppm, while less than 40ppm is used for larvae and juveniles (Table 4 - 1). A stock solution of 60g benzocaine per L (95% denatured ethanol) is prepared for broodstock. At this concentration, 1mL of stock solution is used per litre of seawater in the anaesthetic tub. For larvae and juveniles dosage of anaesthetic can be varied by adding proportionally less of a 40g/L (ie. 40g benzocaine in 1L of 95% denatured alcohol) solution.

Table 4 -1 Dose rates for KGW larvae and juveniles (for stock solution of 40mg/L)

Size of larvae/juvenile	**Dose rate of stock solution per L seawater**
< 50mm	0.3mL
50-70mm	0.4mL
70-150mm	0.5mL
150-200mm	0.7mL
>200mm	1mL

Adults require 1.5mL of a 40ppm stock solution per L of anaesthetic tub volume.

Use half doses to mildly sedate fish rather than to fully anaesthetise them (ie. for tank transfer without inspection or weighing).

4.3.3 Clove oil

Clove oil **(contact NRA for current permit status)** has been found to be a suitable anaesthetic in a range of fish species, including KGW (pers. obs.). Studies on clove oil as an anaesthetic demonstrate it has a calming effect on fish both on entry and during anaesthesia, reducing stress and mortalities

while handling and transporting fish. It has a wide safety margin, maintaining similar effectiveness at both low and high concentrations (Soto and Burhanuddin 1995; Anderson *et al* 1997; Munday and Wilson 1997; Keene *et al* 1998). Clove oil is also easy to handle, safe to fish and to humans during use, has no persistent effects on fish physiology and behaviour, is rapidly excreted or metabolised, leaving no residues requiring withdrawal time, and has shown no problems with repeated exposure (Keene *et al* 1998). Studies noted with some species that the entry into anaesthesia with clove oil was in line with other alternatives, but recovery time was longer.

Clove oil is a cheap product derived from the stem, leaves and bud of the *Eugenia caryophyllata* tree (Keene *et al* 1998). The active ingredient in clove oil is eugenol (4-allyl-2-methoxyphenol) which constitutes about 70–90% of the cloves (Keene *et al* 1998). Eugenol is practically insoluble in water (Budarari *et al* 1989). Clove oil can be partially mixed in warmer water (>15°C). However, only a small amount of eugenol is miscible with water therefore large doses of clove oil are required to get an effective dose of eugenol. Clove oil can be mixed with ethanol as a stock solution to increase the solubility and effectiveness of eugenol (Keene *et al* 1998; Munday and Wilson 1997; Anderson *et al* 1997). At SAASC, sorbitol is used as a solvent as the most effective way to mix clove oil and increase the solubility and effectiveness of eugenol (pers. obs.). Using this method substantially lowers the doses of clove oil required to anaesthetise fish. Sorbitol is freely soluble with water, making it an efficient stock solution.

Procedure for preparing clove oil

Mix 20mL of clove oil with 1L of sorbitol, shake vigorously.

Anaesthetic doses

Anaesthetic doses for juvenile KGW with an average weight of 200g.

- 10ppm (ie. 0.5mL/L) lightly sedates fish (should be left in the solution for about 2–4 minutes).
- 15ppm (ie. 0.75mL/L) lightly anaesthetises fish (should be left in solution for about 2–4 minutes).
- 20ppm (ie. 1.0mL/L) heavily anaesthetises fish (fish should become anaesthetised before 5 minutes).

Recovery

KGW recovered quickly (about 1 minute) after being anaesthetised with clove oil. When using higher concentrations, or when fish have been left under anaesthesia for a longer time, they take longer to recover.

4.4 Broodstock feeds

High quality feeds are crucial in maintaining broodstock and achieving a regular supply of high quality eggs. During the first KGW spawning season (1998) of the project, fish were fed a combination of cockles (*Donax deltoides)* and trawl squid (*Nototodarus gouldi)* supplemented with Barramundi pellets (5mm sinking pellets). Eggs produced throughout the 1998 season were of low quality, characterised by low rates of fertilisation, hatch and survival of larvae to first feeding. As a result, broodstock nutrition was reviewed to formulate a new pellet diet to improve egg quality. The new diet was fed from two months before the following (1999) spawning season, resulting in high quality eggs at a low cost with minimum labour.

Feeding regime

Broodstock are fed once a day. Feed intake will vary according to water temperature and care should be taken not to overfeed. Generally adults are fed between 0.5 -1.0 % body weight/day. KGW will feed at the surface and mid-water when hungry but are generally bottom feeders so feeding should be extended over a number of partial feeds until satiation.

In the months before and during spawning the quantity and quality of feed is important (Section 11.12) and supplementary feeds of squid/prawns and pilchards can also be provided.

It is recommended to keep records and regularly monitor broodstock feeding, as this is often a good indicator of health (Section 11.11.1).

Immediately after capture, new broodstock are fed on wet feed daily. Squid cut into thin strips (about 1–1.15cm width) and pilchards are suitable for KGW. Once new broodstock are acclimatised, healthy and any capture-related abrasions have healed (about 1 month), they can be weaned onto pellets. During weaning, pellets are fed in the morning when fish are hungry to promote acceptance, with squid fed in the afternoon. Progressively, fewer squid is provided over time and pellet feeding increased to compensate.

4.5 Tagging

Female KGW broodstock at SAASC are tagged to allow individuals to be identified during trials. T-bar anchor tags are used rather than PIT (Passive Internal Transporter) tags as anaesthetised or crowded fish can readily be identified by eye.

T-bar anchor tags are designed to be inserted into the muscle of fish in a variety of locations. Attachment of the tag results from the T-end becoming locked behind the bony or cartilaginous structures of the fish. The broodstock are tagged to one side of the body, about 1cm below the 4th or 5th spine of the anterior dorsal fin with the T-bar anchoring between pterigiophores of the skeleton/frame on the opposite side of the fish (Figure 4-6).

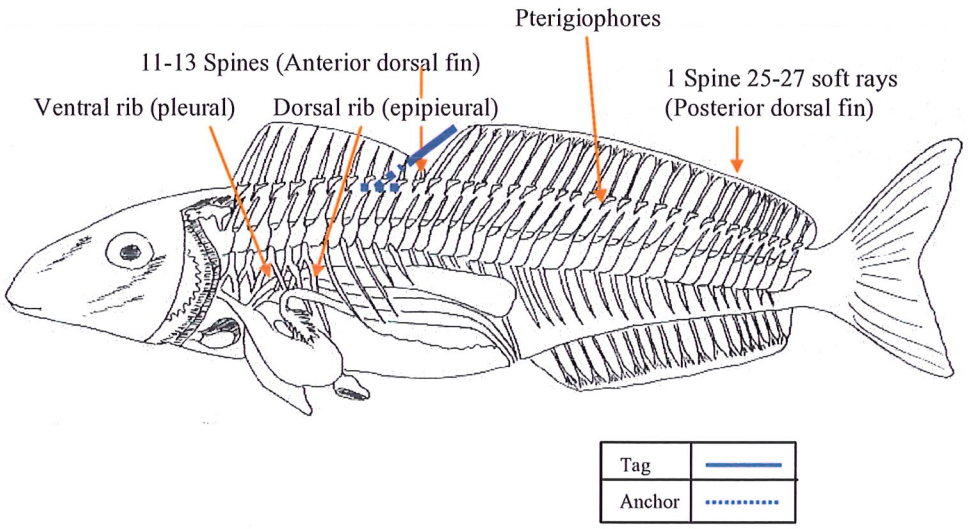


Figure 4 - 6 The T-bar tag anchored between the pterigiophores on the skeleton / frame of a KGW

4.5.1 Procedure for administering T-bar anchor tags

Figure 4 - 7 Locating tag region between the spines on the anterior dorsal fin

Locate the tagging needle in between the spines on the anterior dorsal fin and move the needle down about 1cm from the top of the fish (Figure 4-7).

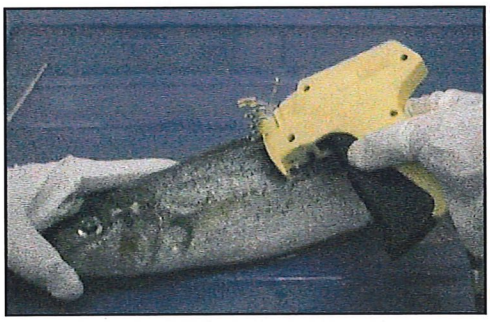


Figure 4 - 8 Inserting needle of tag gun slightly towards the front of the fish

Slightly angle the needle towards the front of the fish (so that the tag will end up pointing towards the back of the fish) Figure 4-8).

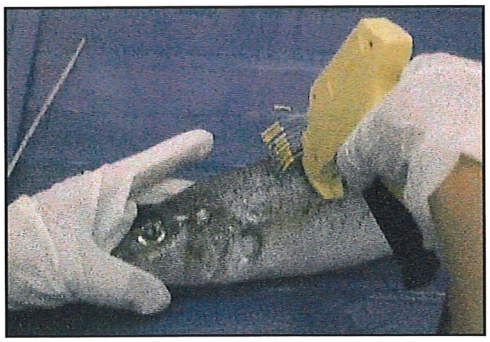


Figure 4 - 9 Inserting needle of the tag gun through the internal frame of the fish

Fully insert the needle diagonally, so that the tag passes through the internal frame of the fish (Figure 4-9).

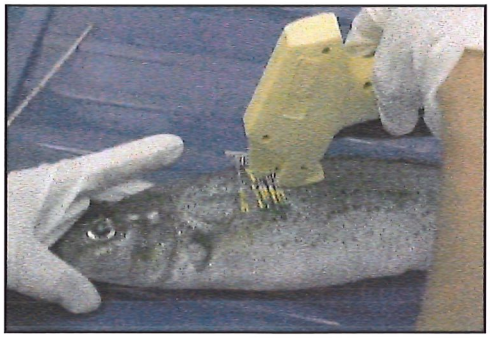


Figure 4 -10 Releasing T-bar anchor tag into the fish

Press the trigger on the gun and turn the gun about 60–90° anticlockwise. Ensure that pressure is maintained on the trigger until the needle is withdrawn from the fish, otherwise the T-bar is forced partly back into the needle by the compressed fish flesh and the tag is sometimes withdrawn into the needle (Dell 1968). When withdrawing the needle, gently hold the tag to ensure it stays in the fish and separates from other tags (Figure 4-10).

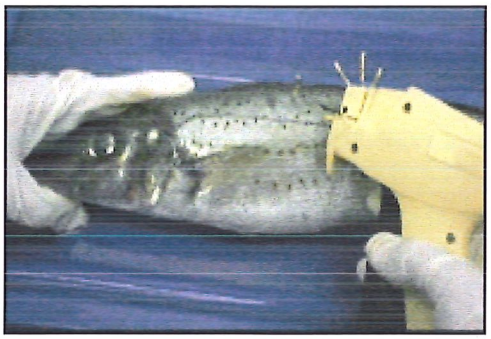


Figure 4 - 11 Checking that the T-bar anchor tag is secured

Once inserted, gently pull on the tag to ensure that it is securely locked behind the pterygiophores. Apply a small amount of Betadine® (antiseptic cream) to the base of the tag to help fight opportunistic infections that could enter the wound (Figure 4-11).

Chapter 5: Egg biology

5.1 Egg development

When spawned, the fertilised eggs of KGW are clear, buoyant and spherical, typically 0.85–0.94mm in diameter with a single oil droplet (0.25–0.26mm diameter). The egg consists of a chorion (shell) separated from the translucent yolk and developing embryo by a fluid-filled perivitelline space (Figure 5-1).

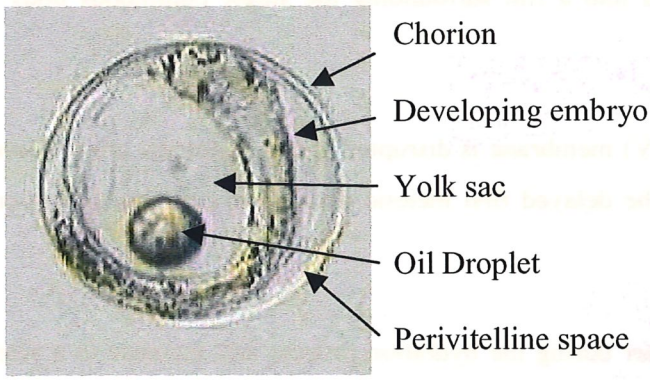


Figure 5 - 1 Components of a developing KGW egg

Eggs are formed within folds of the ovarian tissue of females. The stem cell population of oogonia (germ cells destined to form eggs) undergo mitotic divisions to give rise to secondary oogonia. Secondary oogonia undergo further divisions to transform into primary oocytes (in KGW approximately 40–60µm in diameter) which ultimately will develop into eggs (Figure 5-2) primary oocyte. This process is known as oogenesis.

Primary oocytes continue to develop through the process of pre-vitellogenesis. This stage is marked by accumulation of the cytoplasm, growth of the nucleus and formation of a follicle around the oocyte. KGW oocytes are about 150µm in diameter after completing pre-vitellogenesis.

Oocytes continue to develop through vitellogenesis. During this process yolk protein accumulates as yolk globules which fill the ooplasm and ultimately provide the nutrient and energy reserves required for embryonic development. Lipid droplets containing mainly neural lipids (triglycerides)

also begin to accumulate before the appearance of these yolk globules (West 1990). Towards the end of oocyte development, the small lipid droplets generally coalesce to form several larger droplets which later form the single oil droplet found in fully developed eggs (De Vlaming 1983; West 1990). In KGW, viellogenesis is complete when oocytes attain a size of 400–450µm (Figure 5-2) vitellogenic oocyte. Oocytes remain this size until fish are stimulated by an appropriate environmental cue. Sharp rises in GtH initiate oocyte maturation causing the follicular envelope to release a maturation-inducing steroid, which stimulates final oocyte maturation. Final oocyte maturation is rapid in most teleosts, generally within 24 hours (Selman and Wallace 1989).

In KGW, during final maturation, the germinal vesicle (nucleus) moves from its central position to the animal pole at the edge of the oocyte next to the micropyle (opening through which the sperm enters). After this, the yolk and lipid droplets coalesce separately, forming a single oil droplet and the ooplasm is displaced into a rim surrounding the single continuous body of yolk resulting in increased transparency.

The germinal vesicle (GV) membrane is disrupted and the contents are released into the cytoplasm at the animal pole and the delayed first meiotic division is completed. A second meiotic division begins and is arrested.

Oocytes then absorb water during the hydration process and increase to a size of about 800µm in diameter (Figure 5-2) hydrated oocyte. At this point, the oocytes have matured into eggs which can be fertilised.

Final ovulation takes place, then the oocytes separate from the follicular envelope and the envelope breaks down, releasing the oocyte into the ovarian lumen. Oocytes can then spawn into the water column following appropriate environmental and behavioural cues, where they can be fertilised.

5.1.1 Development series of KGW oocytes from primary oocyte through to hydrated oocyte

Macroscopic view **Microscopic view**

Primary oocyte (unyolked)

Diameter 40–60µm. Macroscopically oocytes cannot be seen without magnification. Ovaries appear glassy, grey-pink and jelly like. At this stage oocytes have a centrally located nucleus that seems large in comparison to the surrounding ooplasm.

Pre-vitellogenic oocyte (unyolked)

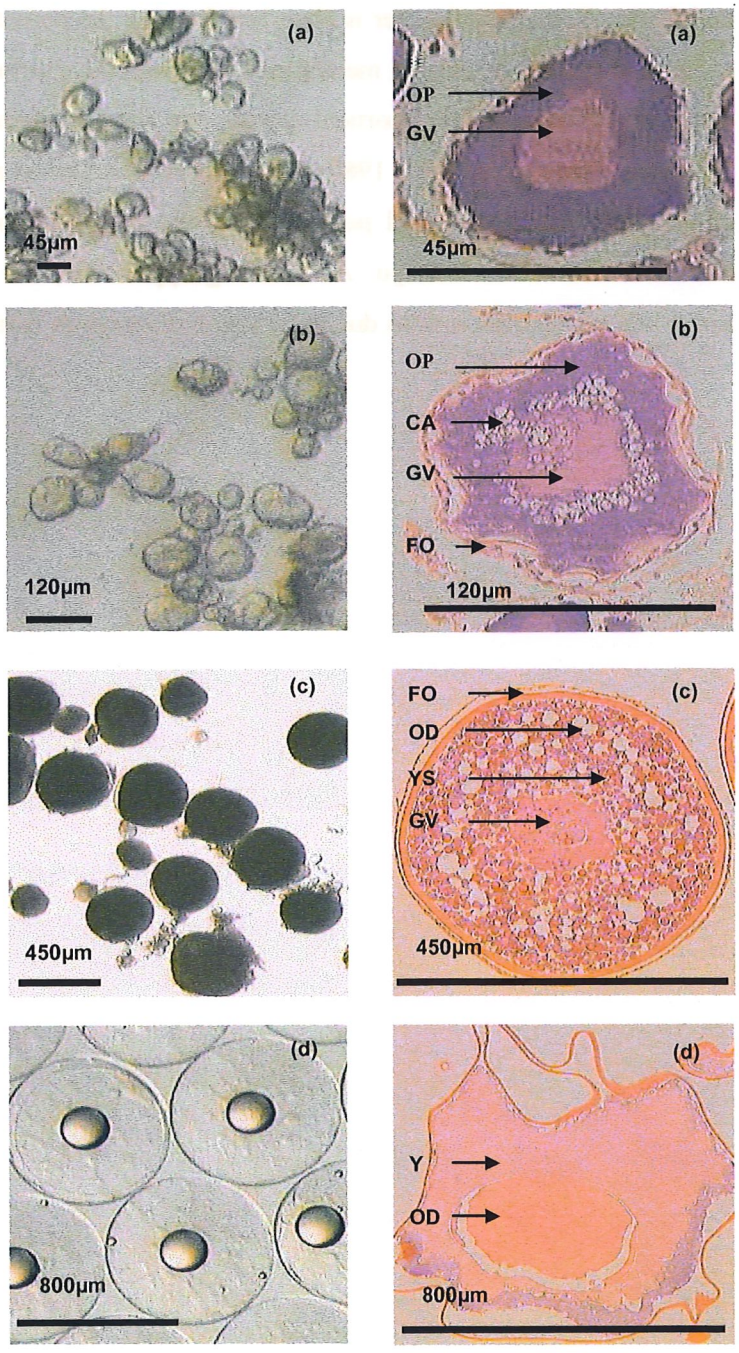
Macroscopically similar to the previous stage although the size range has increased to 120–150µm. Oocytes have cortical alveoli surrounding the nucleus which will later be displaced to the periphery during vitellogenesis.

Vitellogenic oocyte (yolked)

Diameter 400–450µm. Oocytes visible without magnification. Oocytes are yellow in colour with small white/grey centres. Vitellogenic oocytes are marked by scattered yolk and oil spheres that will later consolidate to form a fluid yolk mass and oil droplet.

Hydrated oocyte

Diameter 800-850µm.
Oocytes are fully hydrated and ready to be released into the water column. Yolk globules and oil droplets have coalesced to form a homogenous mass of fluid yolk containing a single oil droplet. The oocyte is transparent.



OP = Ooplasm; GV= Germinal vesicle (nucleus); CA= Cortical alveoli; FO= Follicle; OD = Oil droplet; YS= Yolk sphere; Y= fluid yolk mass

Figure 5 - 2 Development series of KGW oocytes from primary oocyte through to hydrated oocyte (both macroscopic and microscopic view)

5.1.2 Development sequence for KGW eggs from fertilisation until hatch

A development sequence and description of events for KGW eggs from fertilisation until hatch is provided in below. Fertilisation occurs with a single sperm crossing the chorion through the micropyle after which the head of the sperm is incorporated into the cytoplasm (Gilkey 1981) and the chorion hardens and water is absorbed forming the perivitelline space (Blaxter 1988). This is a result of shrinkage of the egg membrane away from the chorion due to osmotic distension caused by discharge of colloids from cortical alveoli into the perivitelline space during the cortical reaction following fertilisation (Laale 1980). The embryo develops as a blastodisc, which engulfs the surface of the yolk from the animal pole until closure of the blastopore at gastrulation and subsequent differentiation of the embryo. As hatching approaches, the embryo becomes increasingly more active and the chorion softens due to secretion of enzymes from hatching glands (Blaxter 1988).

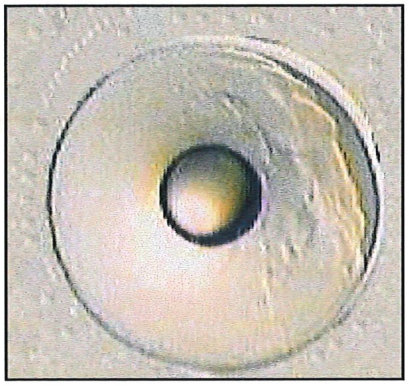


Figure 5 - 3 Stage 1 KGW egg

Stage 1

Undifferentiated (Figure 5-3).

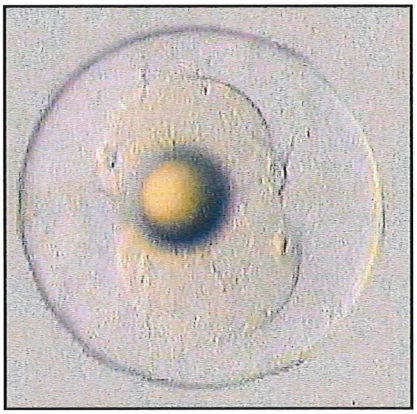


Figure 5 - 4 Stage 2 (2 cell) KGW egg

Stage 2

2 cell stage, 45 minutes after fertilisation (Figure 5-4).

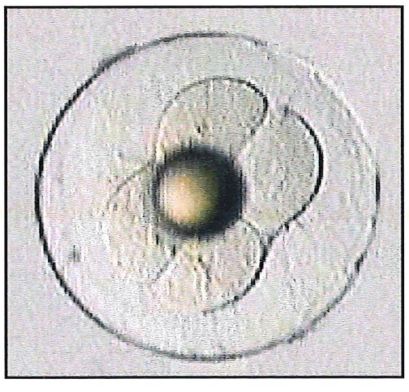


Figure 5 - 5 Stage 2 (4 cell) KGW egg

4 cell stage, 1.5 hours after fertilisation (Figure 5-5).

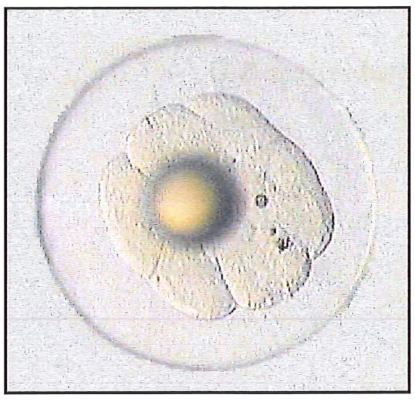


Figure 5 - 6 Stage 2 (8 cell) KGW egg

8 cell stage, 2.5 hours after fertilisation (Figure 5-6).

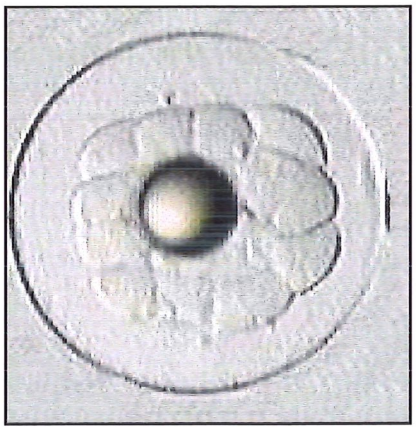


Figure 5 - 7 Stage 2 (16 cell) KGW egg

16 cell stage, 3 hours after fertilisation (Figure 5-7).

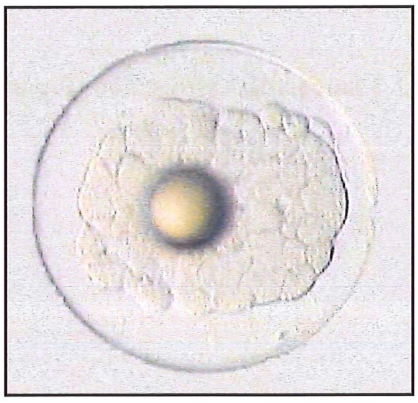


Figure 5 - 8 Stage 2 (32 cell) KGW egg

32 cell Stage, 3.5 hours after fertilisation (Figure 5-8).

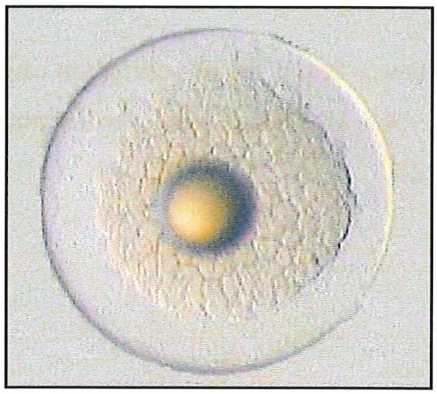


Figure 5 - 9 Stage 3 KGW egg

Stage 3

A multi number of indiscernible cells are still evident; 4.5 hours after fertilisation (Figure 5-9).

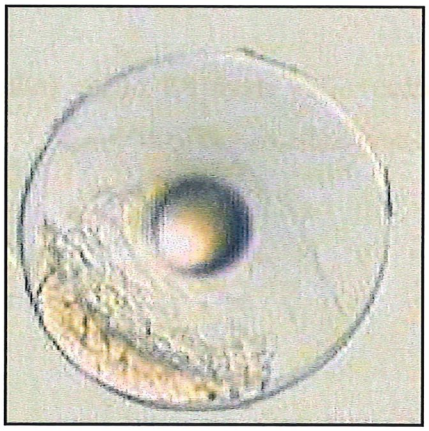


Figure 5 - 10 Stage 4 KGW egg

Stage 4

A distinct blastodisc has formed, consisting of multiple number of small cells; 7.5 hours after fertilisation (Figure 5-10).

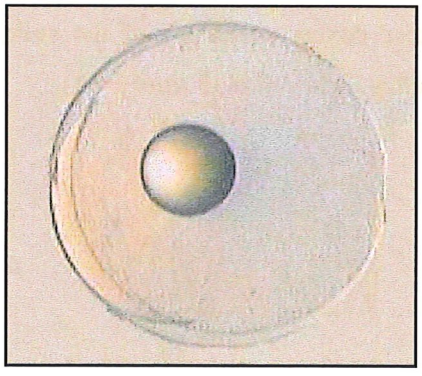


Figure 5 - 11 Stage 5 KGW egg

Stage 5

Blastodisc forming a distinct cap but not yet covering 1/3 of yolk; 12.5 hours after fertilisation (Figure 5-11).

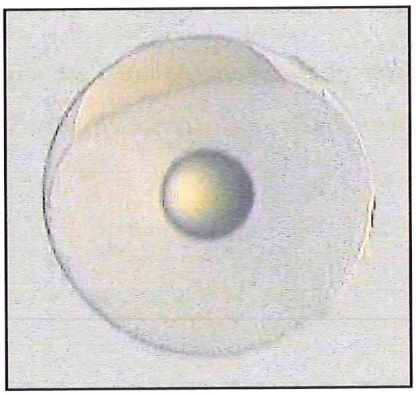


Figure 5 - 12 Stage 6 KGW egg

Stage 6

Blastodisc covers about 1/3 of yolk, germ ring forming; 14.5-15 hours after fertilisation (Figure 5-12).

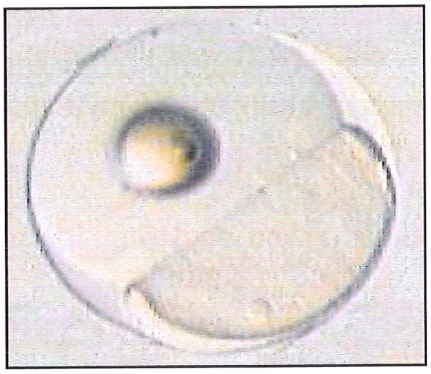


Figure 5 - 13 Stage 7 KGW egg

Stage 7

The blastodisc covers about 1/2 of the yolk; 18.5 hours after fertilisation (Figure 5-13).

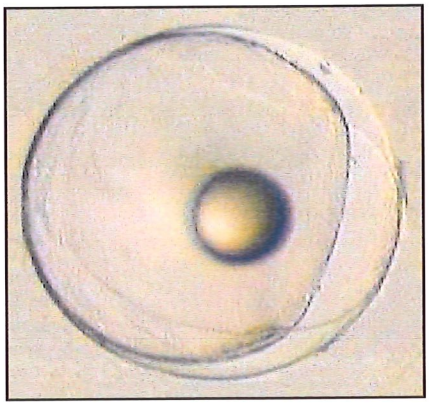


Figure 5 - 14 Stage 8 KGW egg

Stage 8

The blastodisc covers about 3/4 of the yolk; 21.5 hours after fertilisation (Figure 5-14).

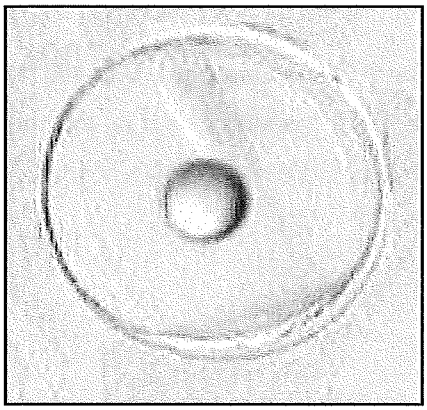


Figure 5 - 15 Stage 9 KGW egg

Stage 9

Rudimentary embryo present, not raised above the yolk surface; 24.5 hours after fertilisation (Figure 5-15).

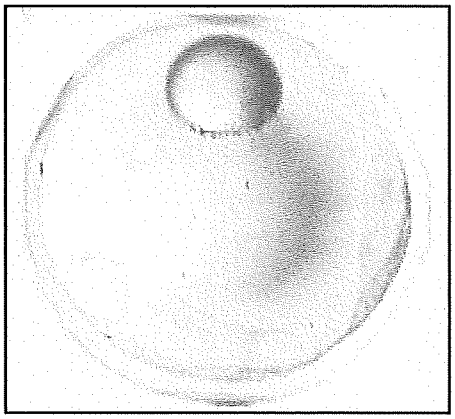


Figure 5 - 16 Stage 10 KGW egg

Stage 10

Embryo more developed and raised thinly above the yolk surface, optical vesicles evident, mysotomes visible, pigment spots present on both embryo and oil droplet; 28 hours after fertilisation (Figure 5-16).

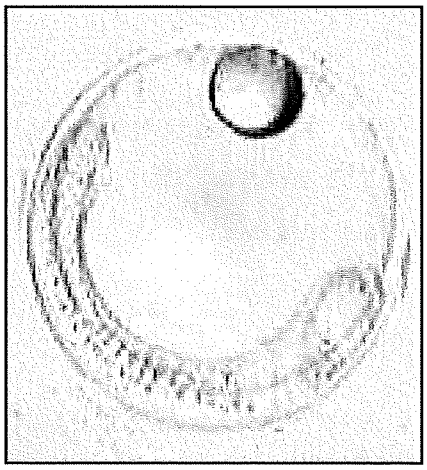


Figure 5 - 17 Stage 11 KGW egg

Stage 11

Embryo well developed, rounded and thick along its entire length, pigment spots very prominent, tail blunt but not yet lifted from the yolk; 35 hours after fertilisation (Figure 5-17).

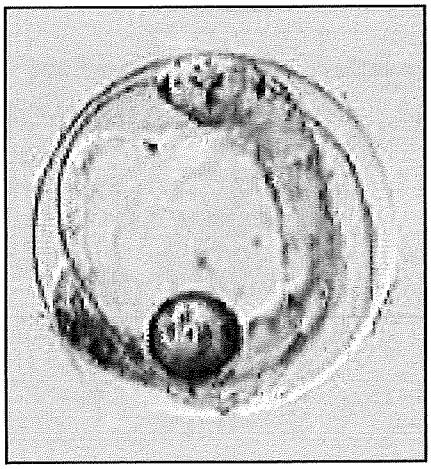


Figure 5 - 18 Stage 12 KGW egg

Stage 12

Embryo about 3/5 around the yolk and its entire length and tail budding starting to lift at the root; 41 hours after fertilisation (Figure 5-18).

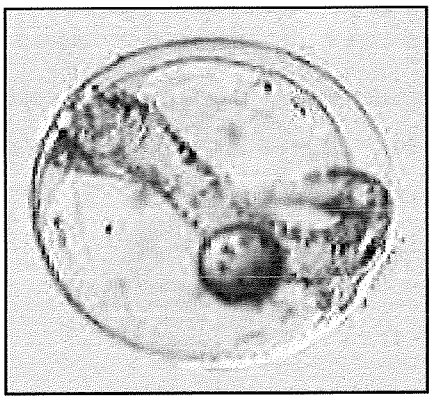


Figure 5 - 19 Stage 13 KGW egg

Stage 13

Tail lifted from yolk and starting to turn away from the mid-line of the embryo. Embryo about 3/5 around the yolk; 43.5 hours after fertilisation (Figure 5-19).

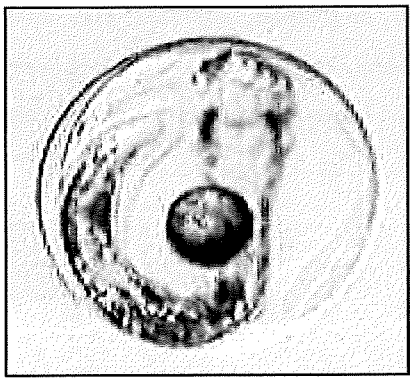
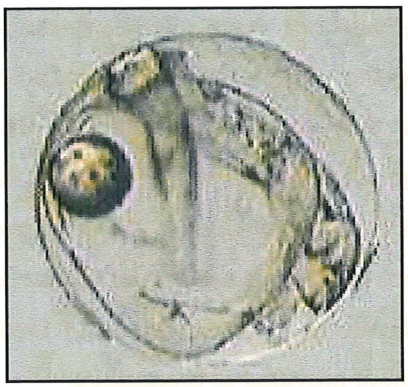


Figure 5 - 20 Stage 14 KGW egg

Stage 14

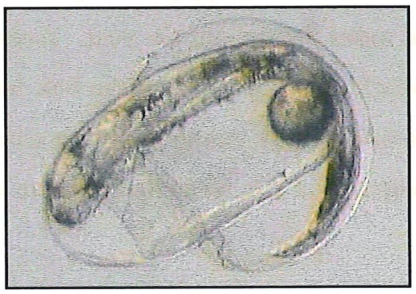
Tail pointed away from the mid line of body halfway along the embryo; 48 hours after fertilisation (Figure 5-20).



Stage 15

Embryo orientated at one pole of the yolk sac – off centre from the line of the oil droplet; 52 hours after fertilisation (Figure 5-21).

Figure 5 - 21 Stage 15 KGW egg



Hatch

The embryo is fully developed and breaking free from the chorion of the egg; 54 hours after fertilisation (Figure 5-22).

Figure 5 - 22 Hatching of KGW larvae

Note: Eggs in this development series were incubated at 18°C. Egg development stages as per Fowler (2000).

5.1.3 Energetics of egg and larval development

Once hatched, larvae are dependent on endogenous food reserves to sustain their growth and development to first feeding. To date, no known studies have been conducted on the energetics of egg and larval development for KGW. Studies on other marine finfish suggest that energy substrates used during development may vary between species. Lipid is the energy substrate preferred by developing eggs and larvae of dolphin fish (*Coryphaena hippurus*) with protein metabolised during hatching (Ostrowski and Divakaran 1991). Ronnestad *et al* (1994) suggest that the free amino acid (FAA) pool found in the yolk sac of gilt head seabream (*Sparus auratus*) provides the energy source for development during the egg stage and after hatching. Neutral lipids derived from the oil droplet are the dominant energy source until the onset of first feeding. Finn and Fyhn (1995) found for Atlantic cod (*Gadus morhua*) a species which has eggs without an oil droplet,

that eggs and larvae use predominantly FAA's as the energy substrate during development with additional contribution from lipid after closure of the blastopore. The same authors found that turbot (*Scopthalmus maximus*), which have eggs with an oil droplet, use predominantly FAA's during the embryonic stage and lipid during the yolk sac stage of development. The neutral lipid triacyglycerol (TAG) and FAA are also the energy substrates used during the embryonic development of red seabream (*Pagrus major*), which have eggs with a single oil droplet (Seoka *et al* 1997). In all cases the energy required for development of larvae until the beginning of feeding must be provided in the egg, which is ultimately reliant on nutrition provided to broodstock.

5.1.4 Egg production for research and hatchery operations

Both research and commercial hatchery operations require a controlled supply of high quality fertilised eggs. If this can be achieved at regular and planned times throughout the year, research can be accelerated and commercial hatcheries can provide juveniles to on-growers at the best times for growth and market opportunities. Controlled egg production can be achieved in the natural spawning season and outside this through environmental manipulation (ie. water temperature and day length) and hormone-induced spawning procedures.

5.2 Egg production during the natural spawning season

5.2.1 Spawning season

During this project, best results have been achieved during the natural spawning season. The first spawning season of captive broodstock after capture (1998) was shorter than in the wild, from mid April (14/4/98) until late may (28/5/98). Egg production was very limited (734,500 eggs in total) with a low viability (17.69%) (Figure 5-23). Few females spawned, and those that did seemed to be experiencing problems synchronising with males. Males were producing milt from April until June.

During spawning of captive broodstock the second year after capture (1999), the spawning period was similar to the wild spawning season, from early April (6/4/99) until early June (8/6/99), with the peak in late April - early May. Egg production increased substantially (Figure 5-23) but not sufficiently for commercial production. Average daily egg production was 59,505 (from 38 females over a period of 63 days) with a total of just under 4 million eggs for the spawning season. The viability of eggs spawned increased from the previous spawning period to 55.32%. The percentage of females spawning at one time increased this year to 16.5%. Males produced milt from early April to late June 1999, similar to 1998.

It was thought that the spawning fraction and viability would increase with further domestication. Results from the third spawning season (2000) demonstrate that this was not the case, indicating that natural spawning of wild caught domesticated broodstock without induction cannot be relied upon to produce eggs for a commercial hatchery. The viability of eggs spawned during the 2000 spawning season was 44.16% with the percentage of females spawning at any one time down to about 7.6%. Average daily egg production was 71,869 (from 82 females over a period of 50 days) with a total of 3,593,470 eggs for the spawning season. The spawning period was from April (13/04/00) to early June (02/06/00), shorter than the 1999 season. Males produced milt from about early April to mid-June. It seems likely that the compressed spawning season in 2000 can be attributed too higher than normal temperatures in March–April (seawater temperature, 21°C on 31/03/00) delaying the onset of spawning. An unusually cold spike in late May (28/05/00) (seawater temperature, 14.1°C) appeared to cause spawning to end. This notion is in line with the previous spawning season results, where spawning began and ended when the seawater temperature was 19.1°C and 14.8°C respectively. These results suggest that KGW broodstock require seawater temperatures between 19°C and 15°C for spawning.

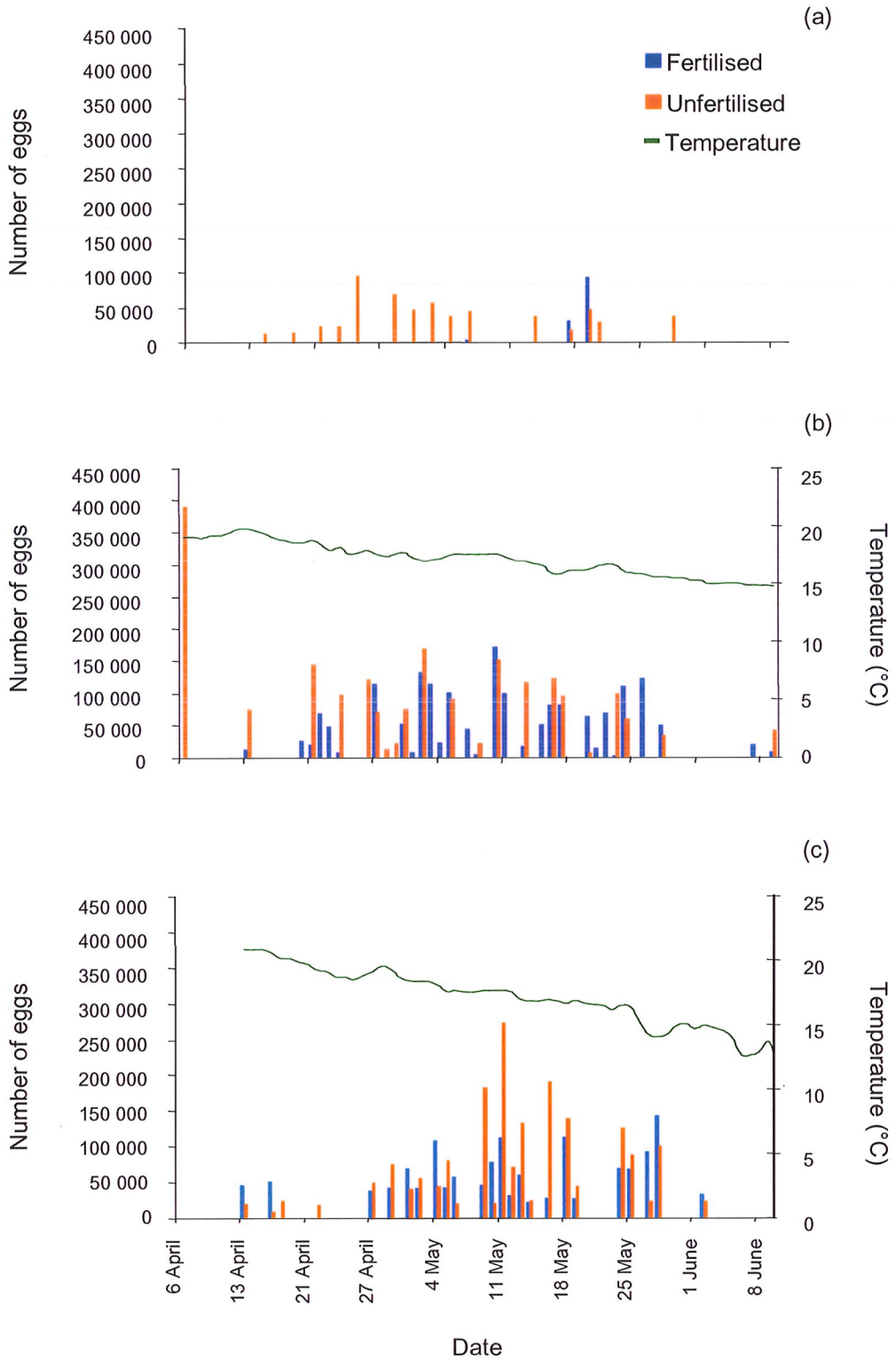


Figure 5 - 23 Egg production during the natural spawning season the first (1998), second (1999) and third (2000) year after capture

5.2.2 Spawning frequency

KGW appear to be multiple batch spawners with asynchronous development and indeterminate fecundity (Fowler *et al*, 1999). This means that at any one time during the spawning season there are eggs of all development stages in the ovary and not all maturing oocytes undergo the final stages of maturation and spawning at the same time, only a batch of matured oocytes. From these studies, it appears that KGW females spawn several times, either over consecutive days, or at least within a few days of each other, and the amount of eggs spawned over a spawning season is not fixed before the spawning season.

5.2.3 Fecundity/spawning numbers

Estimates of batch fecundity in wild fish range from 5,250 to 152,191 hydrated oocytes per ovary, proportional to fish size. Estimates of annual fecundity (ie. the numbers of eggs spawned per female per year) in the wild, calculated by multiplication of spawning frequency and batch fecundity, range from 110,250 to 6,087,640 eggs depending on fish size (Fowler *et al* 1999).

5.2.4 Spawning times

Studies conducted on KGW in the wild indicate that spawning occurs at about 3pm in the afternoon. Captive broodstock maintained in outdoor tanks spawned at about dusk, which was about 6pm in mid-spawning season and slightly earlier closer to the end of the spawning season (pers. obs.).

Chapter 6: Controlled egg production

6.1 Overview of egg production options

There are several different options available to achieve a supply of eggs from KGW broodstock (Figure 6 - 1). These include implanting broodstock with slow release cholesterol/cellulose LHRHa pellets, administering an intraperitoneal LHRHa injection or allowing fish to undergo a natural tank spawning.

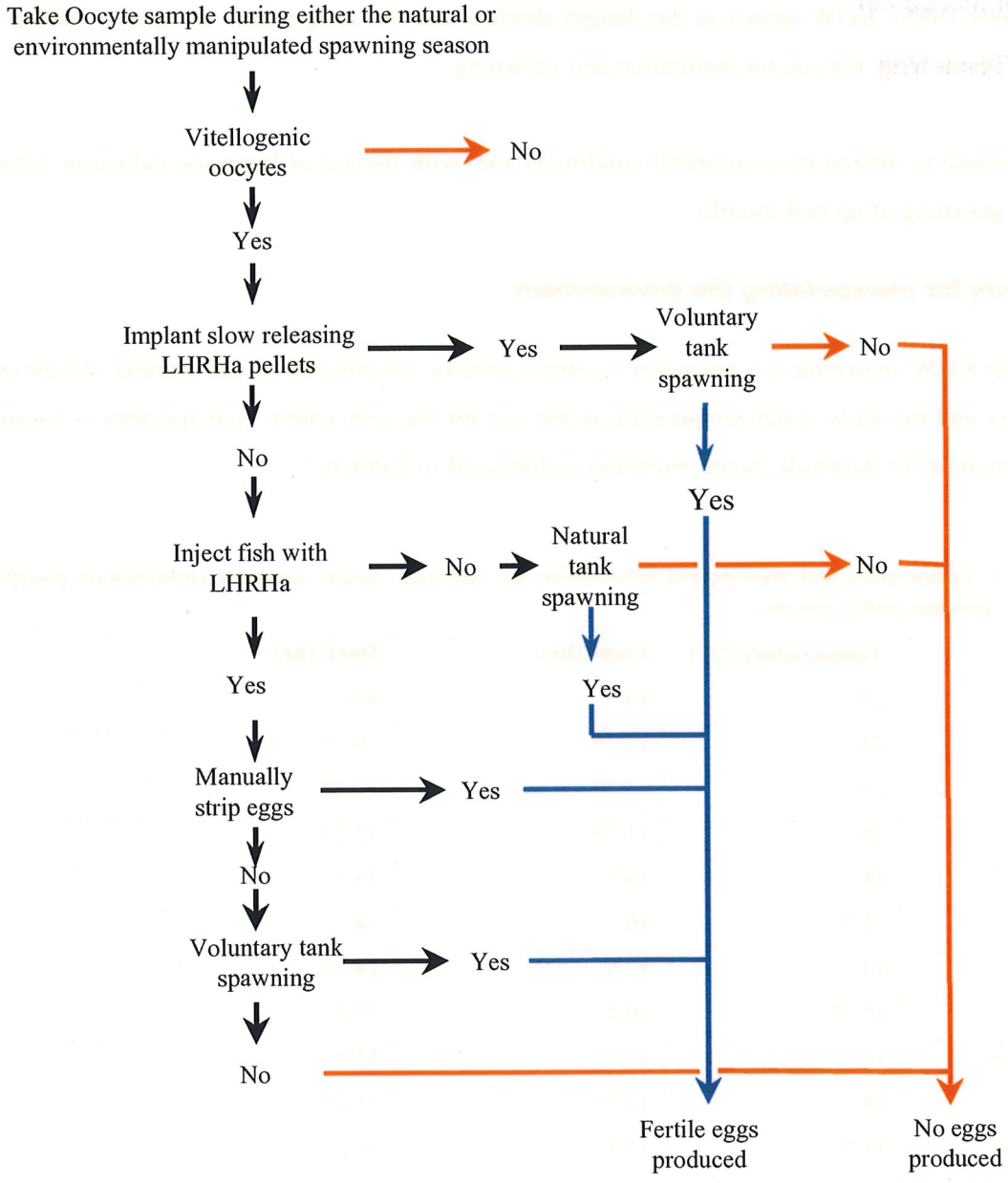


Figure 6 - 1 Overview of egg production options

6.2 Environmental manipulation

To produce a year-round supply of eggs, broodstock can be maintained under an altered photoperiod and water temperature regime promoting out-of-season spawning.

In some fish (such as carp), maturation is stimulated by temperature, while for others reproduction is cued by light, spawning at specific phases of the annually changing cycle of day length (Bromage and Roberts 1995). KGW spawn as day length shortens and the temperature decreases. Neither in isolation seems to be the cue for maturation and spawning.

KGW respond to altered environmental conditions and, with the aid of hormone induction, allows advance spawning of up to 4 months.

Procedure for manipulating the environment

To indulge KGW spawning it is necessary to obtain specific information on the number of light and dark hours and the daily water temperature in the sea for the area where your hatchery is located. The information for Adelaide, South Australia is displayed in Table 6-1.

Table 6 - 1 Temperature and photoperiod information for Adelaide, South Australia (Information modified from http://geodesy.auslig.gov.au)

Month	Temperature (°C)	Light (hr)	Dark (hr)
January	23	14.5	9.5
February	24	13.75	10.25
March	23	12.75	11.25
April	20	11.75	12.25
May	18	10.5	13.5
June	15.5	10	14
July	13	9.75	14.25
August	12.75	10.5	13.5
September	13	11.5	12.5
October	16	12.75	11.25
November	18.5	13.5	10.5
December	21	14.25	9.75

The peak of spawning during the natural season needs to be identified. For KGW in South Australia, is late April.

Also, identify the time that you would like spawning to occur, for example, late November (4 month advance spawning), and then identify the month that you are in (for example, June).

Draw up a blank chart with all the months listed. Add the information for April to November on the blank chart and put in the information for the month you are in (for example, June) in June on the blank chart.

The 3 months (approximately) leading up to spawning in the natural season should be maintained (ie. not compressed) where possible. Therefore, on the blank chart, put the information for March in October; the information for February in September and the information for January in August.

Throughout the remainder of June and the month of July, the temperature and photoperiod must be compressed to reach 23°C with 14.5 hours light and 9.5 hours dark (actual data for January) by the end of July (Table 6-2).

Table 6 - 2 Example of manipulated photoperiod and temperature regime

Month	**Temperature**	**Light**	**Dark**
June where we are now ➜	15	10	14
July	Temperatures and light and dark cycles must be compressed during July to reach August's predicted temperature and photoperiod		
August	23	14.5	9.5
September	24	13.75	10.25
October	23	12.75	11.25
November	20	11.75	12.25

Once the photoperiod and temperature has been manipulated so that it is exactly 4 months in advance of the actual annual cycle, these broodstock can be maintained under this altered regime and will spawn in November each year (Figure 6 - 2).

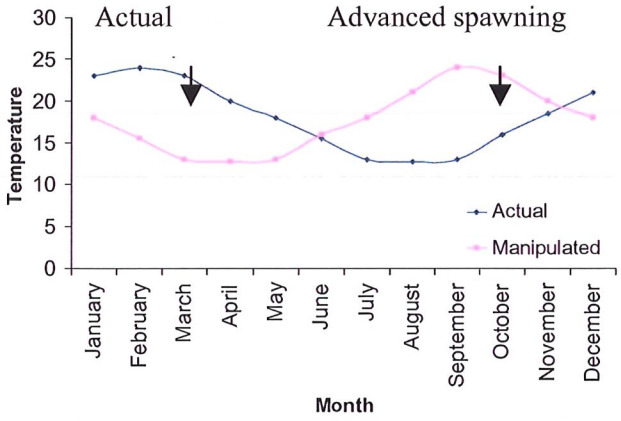


Figure 6 - 2 Graph showing the 'actual' seawater temperature (°C) in Adelaide over the period of a year and the manipulated seawater temperature that would be required to advance spawning by 4 months at this location.

6.3 Inducing spawning

Broodstock of many fish species of interest to aquaculture exhibit some degree of spawning dysfunction when maintained in captivity which is most often observed as females completing vitellogenesis but failing to undergo final oocyte maturation (FOM) which precedes release of eggs (ie. spawning) and fertilisation (Mylonas and Zohar 2001). Such spawning dysfunction most likely results from the combination of chronic stress associated with captivity and the absence of appropriate environmental stimulus or cues. In these cases the use of hormone induced spawning methods is required.

Throughout this project wild caught male King George whiting broodstock held in captivity completed spermatogenesis and where "running" throughout the spawning season while females completed vitellogenesis but few individuals progressed through FOM and spontaneous spawning was unreliable. During each natural season the spawning fraction (number of fish participating in spawning) was typically low 7.6% (2000 spawning season) with subsequent low numbers of eggs collected mean 72,000/day (2000 spawning season). In addition the frequency of spawning was erratic and fertilisation rate varied between 0% and 90% but was typically less than 50%. This scenario is another example of the most commonly observed type of spawning dysfunction in captive finfish broodstock where FOM is an unpredictable or absent (Mylonas and Zohar 2001). These factors together suggest that for this species greater control over egg production (ie. timing,

quantity and quality) is required for a commercial hatchery operation. Hence investigations to develop suitable induced spawning methods were undertaken in 2000 and 2001 and are continuing.

It may be that the problem of limited spawning of captive King George whiting broodstock will diminish after a number of generations of hatchery reared fish have been cultured through to maturity. This has been the case with species such as gilthead seabream, which initially required hormone induction followed by manual stripping and fertilisation procedures. As "domestication" has progressed, the need for external hormone intervention in spawning has decreased to the extent that most broodstock spawn daily over three month season (Barbardo *et al* 1997). A similar trend is emerging with the culture of striped bass (*Morone saxatilis*) with FOM being observed more often but hormone treatments are still used to induce and synchronise spawning (Zohar and Mylonas 2001).

Current methods for spawning induction of broodstock are based on an increasingly better understanding of hormonal control of reproduction of finfish. Early induction procedures used relatively crude ground pituitaries and pituitary extracts (eg carp pituitary extract) taken from spawning fish containing varying levels of gonadotropin (GtH). This method was improved with the increased availability of human chorionic gonadotropin (hCG) which is still widely used and effective in a range of species most likely due to it's long retention time in circulation (Ohta and Tanaka 1997). Modern methods use potent synthetic forms of gonadotropin releasing hormone (GnRHa's) used alone or in combination with domperidone (eg. Ovaprim®) which acts to negate the effect of the natural GtH releasing inhibitor, dopamine. GnRHa's act a high level in the hypothalamus-pituitary-gonad axis and presumably better integrate the sequence of hormonal events through stimulation of natural GtH and other hormones needed for complete FOM (Zohar and Mylonas 2001).

Of importance to aquaculturalists is the understanding that reproductive development in finfish is divided into two main stages.

- Stage 1 Vitellogenesis and spermatogenesis - proliferation, growth and differentiation of oocytes (developing eggs) and sperm.
- Stage 2 Final oocyte maturation and spermiation – maturation and preparation for release of oocytes and sperm (Mylonas and Zohar 2001).

During vitellogenesis yolk proteins accumulate in the oocyte which grows to a species specific diameter after which no further increase takes place until appropriate spawning stimulus occurs. This is the case for most captive broodstock, which undergo vitellogenesis but fail to progress to

FOM and spawning. During FOM a set of relatively fast developmental events occur within the oocyte. The nucleus migrates to periphery and breaks down releasing its contents into the area below the micropyle (site of sperm entry). During this process the accumulated yolk globules and oil droplets coalesce to give the oocytes a transparent appearance. Accompanying these changes there is a rapid increase in size diameter due to hydration (uptake of water) which may increase oocyte volume by 300-400% in some species. During or soon after FOM the oocyte is ovulated into the central lumen region of the ovary from where it is expelled during spawning (Guraya 1986; Selman and Wallace 1989).

Recent findings have identified the existence in finfish of two or three different forms of GnRH which all induce release of GtH. In addition there are two different forms of GtH which provide control of each of the two stages of development described above (Mylonas and Zohar 2001). It is generally accepted that GtH I (follicle stimulating hormone, FSH) is primarily responsible for regulating vitellogenesis and spermatogenesis while GtH II (lutenising hormone, LH) regulates FOM and spermiation. When spawning conditions prevail at the completion of vitellogenesis there is a surge in GtH II which stimulates release of a maturation inducing steroid which acts to induce FOM (Zohar and Mylonas 2001; Mylonas and Zohar 2001).

It appears that most reproductive dysfunction of captive finfish species, including KGW, may be attributed to failure of vitellogenic oocytes to proceed through FOM. This can be overcome by administering GnRH (ie. LHRHa or Ovaprim) to provide the hormone surge needed to stimulate release of GtH II and MIS required to complete FOM. This is the direction of research to be followed in order to develop methods to provide regular supply of commercial quantities of good quality eggs from captive KGW broodstock. In addition first generation hatchery reared broodstock will soon be approaching maturity to allow assessment of improved egg production over subsequent generations of domestication.

6.3.1 Administration of hormone

Hormone treatments can be administered as:

- intra peritoneal or intra muscular injections, and
- intra muscular implanting of slow release pellets.

To date, hormone treatments have not been effective in promoting spawning of KGW with unyolked (immature) oocytes and consequently should only be used on fish approaching or having already

completed vitellogenesis (Section 5.1.1). When attempting to assess the reproductive stage of KGW by examining oocytes, consider only the largest oocytes, although smaller immature oocytes may be present (this is characteristic of multiple batch spawners).

6.3.2 Intraperitoneal injections

Typically injection of GnRha will induce a relatively short surge in levels of GtH which may be sustained for long enough to stimulate the final stages of maturation. Administering LHRHa (10-20µg/kg) as an intraperitoneal injection can induce final stages of maturation and spawning of KGW females after 52 hours.

While it is possible to strip fish after an intraperitoneal injection, eggs collected to date through this process have mostly been of poor quality, although the numbers produced and control of production were as required for commercial hatchery operations. The preferred method at SAASC was to collect eggs via tank overflow after a voluntary spawning from fish injected with lower doses of LHRHa (10µg/kg bw), as they were generally high quality and viable.

Procedure for preparing intraperitoneal injections

LHRHa vials (1mg) are diluted into injectable saline (0.9% saline) to provide a convenient injectable dose, which can be adjusted to suit the size range of the fish injected. Solutions of LHRHa in a saline solution can be injected directly into the peritoneal cavity of fish. To suit the average KGW broodstock size (500–1000g), we used a 1:20 dilution of LHRHa:saline (assuming that complete 1mg vials of LHRHa ([des-gly10, D-trp6, pro9-ethylamide] LHRH analogue, Peptech Ltd) are used).

Example

Desired dose = 10µg LHRHa/kg body weight

In a 1 LHRHa :20 saline dilution

10µg LHRHa is in 0.2mL of solution

∴ 5µg LHRHa is in 0.1mL of solution (the dose for a 500g fish)

(Section 11.9 for dose tables)

Requirements

- 1mg vials of LHRHa.
- 20mL injectable saline (0.9%).
- 1mL hypodermic syringes.
- 30 gauge 1/2 inch needles.
- Glass sealed container.

Procedure

- Measure 20mL of saline into sealed glass container.
- Connect needle and syringe.
- Draw up pre-measured saline solution into syringe and add to a 1mg vial of LHRHa. Mix and retract contents of each vial back into glass container with saline solution. Repeat until all hormone is mixed thoroughly into saline solution.
- 1mL of mixed solution is drawn up into the syringe ready to be injected into fish.
- Remaining solution can be labelled and stored in the refrigerator for future use. Note: the manufacturer recommends the storage life of LHRHa. Peptech Limited suggests that unless the material is to be used within a month of receipt, storage should be at below minus 20°C. For use within a month, storage at less than 5°C should be adequate. Storage under both conditions should be desiccated as peptides such as LHRHa are sensitive to moisture absorption, which will adversely effect their stability and peptide content. For this reason our recommendation is that the minimum required should be made up and used and the remaining solution disposed.

Procedure for administering intraperitoneal injections

- Capture fish and anaesthetise as mentioned in other chapters (Section 4.2.1 and 4.3).
- Weigh fish in grams and calculate the amount of hormone solution required according to 'Dose tables' (Section 11.9).
- Lay fish flat on a bench with the dorsal side facing towards you.
- Draw up the solution in a syringe ensuring there are no air bubbles trapped in the syringe.
- Position the needle half way between the head and anal pore on the dorsal side of the fish underneath a scale (Figure 6-3).
- Gently insert the needle into the peritoneal cavity of the fish (approximately 0.5cm) on a 45° angle pushing on the plunger to administer the required amount.

- Withdraw the syringe.
- Allow fish to recover in fresh seawater.

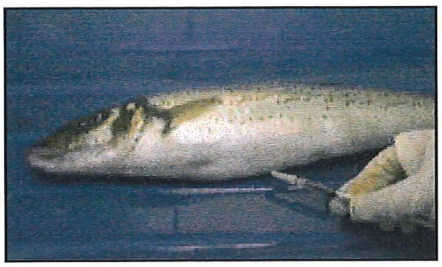


Figure 6 - 3 Administering intraperitoneal injections into the peritoneal cavity of KGW

6.3.3 Implant pellets

LHRHa can be administered in solid, cylindrical (2mm diameter x 3-8mm long) cholesterol/cellulose pellets which are implanted intra muscularly using an implanter. This treatment method provides sustained release of the hormone to stimulate and elevate plasma GtH levels for days or weeks. Cholesterol implants are relatively easy to prepare and are not expensive although GnRHa release can be variable (Sherwood *et al* 1988).

Initial doses of LHRHa as a pellet 90% cholesterol and 10% cellulose were based on previous snapper (Pagrus auratus) research at SARDI (25µg LHRHa/kg body weight (bw)). These were found not to be suitable in KGW, causing gonad development with no release of eggs and some mortalities due to females becoming 'egg bound'.

Lowered doses (ie 10-15 µg/kg bw) of LHRHa pellets were trialed during out-of-season spawning, and while lower doses of hormone seemed to have a more positive effect, trials were discontinued due to disease complications. *Exophiala salmonis*, a fungal pathogen, was at this time infecting fish and causing mortalities. Additional opportunistic infections *Pseudomonas aeruginosa* and Vibrio *alginolyticus* occurred at the site of pellet administration after implantation. It is suggested that the stress associated with these infections may have compromised the attempt to promote reproductive development through environmental manipulation.

The effect of a priming dose pellet with a follow-up dose as a pellet during out-of-season spawning was also trialed. For all treatments, the dose rate of the first implant was 10µg LHRHa/kg body weight (bw) pellets (90% cholesterol), prepared following the method described by Lee *et al* (1986).

The dose rate of the 2nd implant was 10, 15 and 20µg LHRHa/kg bw for treatments 1, 2 and 3 respectively, with one tank serving as a control group (untreated).

Again, disease problems were thought to compromise results as although eggs were induced the numbers were low and fertilisation rates were negligible, so no treatment could be identified as optimal. No spawning was recorded for control (untreated) fish.

Procedure for preparation of slow release luteinizing hormone (LHRHa) pellets

This procedure is based on Lee *et al* (1985) with pellet formulation altered to provide 10% copha binder with desired hormone dosage (per kg) contained in 5mg of final pellet. This pellet formulation procedure also assumes that complete 1mg vials of LHRHa ([des-gly^{10}, D-trp^{6}, pro^{9}-ethylamide] LHRH analogue, Peptech Ltd) will be used. This preparation procedure will provide a convenient size of pellet which can be adjusted to suit the size range of fish to be implanted (ie. 500g–1kg). If smaller fish or larger fish are to be implanted, the pellet formulation should be adjusted to provide the required dosage in a convenient size pellet.

Example

Desired dosage = 10µg LHRHa/kg body weight.

Pellet formulation to give 10µg LHRHa per 40mg of pellet ie. the amount selected to be administered to each 1.0kg of fish (size range 500g–1.0 kg).

Ingredients	**(mg)**
LHRHa	1 (1,000µg)
Cholesterol	3,599
Copha (10%)	400
Total	4,000

Requirements

1mg vials of LHRHa.

Cholesterol powder.

Copha.

250µL auto-pipette.

1000µL auto-pipette.

250µL tips (yellow).

1000µL tips (blue).

Drying oven.

600mL glass beaker.

150mL glass beaker.

1mL syringe.

Pipette bulb.

25 gauge x 19mm needle.

Electronic scales accurate to 0.0000g.

Glass evaporating dish.

Small mortar and pestle.

Small artists paint brush.

Microscope slide.

Pellet press.

Ethanol.

Distilled water.

Spatula.

Glass rod.

2.0mm pin punch.

3.0mL plastic vials.

Proceedure

1. Measure required amount of cholesterol powder onto glass evaporating dish.
2. Using pre-set 250µL auto-pipette, add 100µL 50 % ethanol + 50% distilled water to each 1mg vial of LHRHa. Mix and retract contents of each vial into a 3.0mL plastic sample vial.
3. Repeat step 2.
4. Using 1000µL auto-pipette, drip contents of 3.0mL vial onto surface of cholesterol powder. Mix thoroughly using a glass rod to form thick paste.
5. Evaporate ethanol in drying oven for 2 hours at 30°C. When dry, transfer contents to mortar and pestle. Remove all contents using small brush. Mix/grind thoroughly then return powder to evaporating dish.

6. Transfer 1cm block of copha to 150mL glass beaker and float this in hot water in 600mL beaker. When copha is molten draw into 1mL syringe (without needle) with pipette bulb attached. Attach 25 gauge needle and warm in beaker.
7. Place evaporating dish containing dried cholesterol/LHRHa powder over top of 600mL beaker of hot water. Allow to warm for 2–3 minutes then transfer to electronic scales and drip copha onto mixture until required amount has been added.
8. Transfer contents onto pre-warmed sheet of glass placed over 600mL beaker of warm water. Mix thoroughly for 5–10 minutes using microscope slide to divide and cut mixture.
9. Once thoroughly mixed, weigh 400mg of mixture and transfer onto assembled pellet mould and distribute into selected holes using microscope slide. Place remaining mixture into a sealed refrigerated container (clearly labelled) for additional batches.
10. Press contents of each hole using a 2.0mm pin punch.
11. Repeat steps 9 and 10 until all powder has been pressed into holes.
12. Disassemble pellet mould and carefully poke out pellets using a pin punch.
13. Weigh each pellet to the nearest 1.0mg and transfer to appropriately labelled 3.0mL vials.
14. Store sealed vials in refrigerator until required.
15. For additional batches repeat steps 9–14.

Procedure for implanting pellets

Pellets are implanted into thick muscle behind the head, mid-way between the lateral line and below the first dorsal spine (figure 6 - 4), slightly anterior to the spine. Pellets are inserted using a pellet implanter into the anterior dorso-lateral musculature until the skeletal frame is felt (approximately 2-3cm). The implanter is constructed from 2-210 stainless steel tubing with wooden handle drilled out to load pellets. A wire rod (1.5mm in diameter) is used as a plunger to push pellets to the base of the hollow tube.

1. Before administering pellets, measure the weight of the fish, to obtain the correct dose of hormone.
2. Assemble implanter with internal plunger. Locate the incision point, on the side of the fish above the mid-line approximately a third of the way along the body from the head. Place the scalpel under the scale at an angle (Figure 6-4), then bring to vertical a vertical position and make a small (4-6mm) incision sufficient to insert the implanter (figure 6-5).

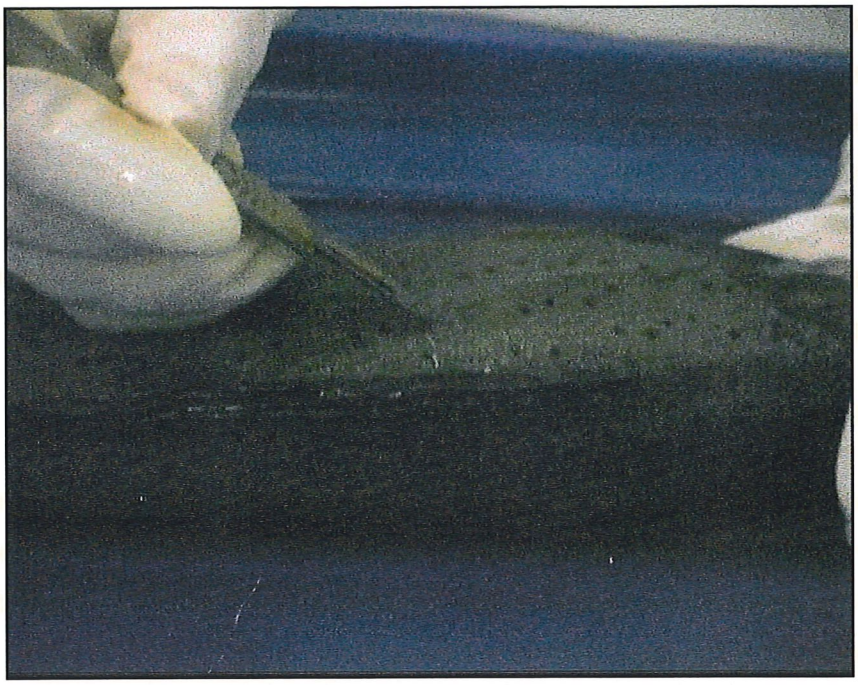


Figure 6 - 4 Locating the incision point for inserting LHRHa slow release pellets

3. Place the flat side of the rod firmly against the scalpel and insert it into the incision (*see* figure 6 - 5). With a moderate amount of pressure, push the rod into the flesh until it comes to rest against the frame of the fish.

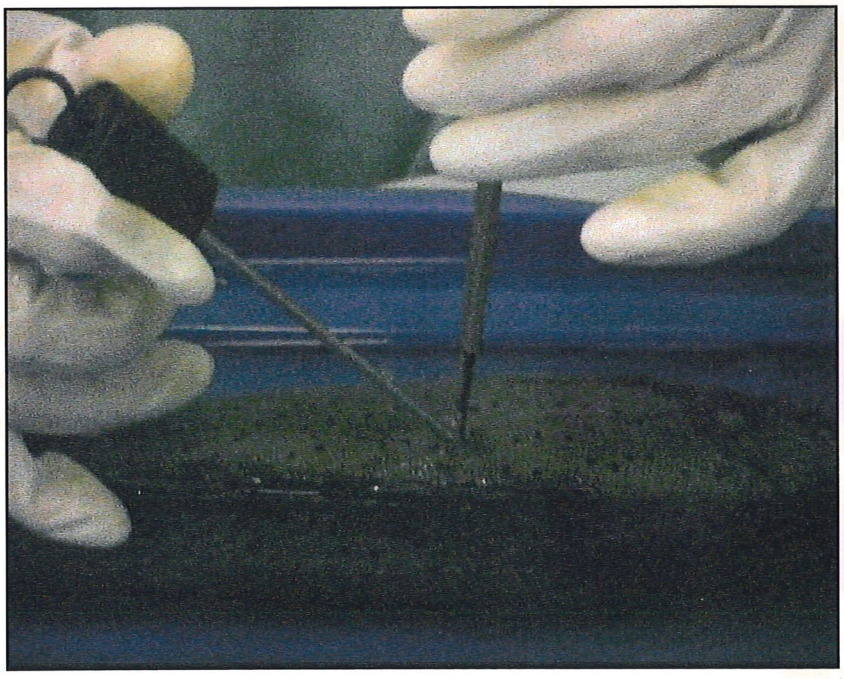


Figure 6 - 5 Inserting the implant rod into the flesh of the fish

4. Remove the internal rod from the exterior casing and load the pellet/s into the handle and tap to position them into the top of the implanter tube. Insert the rod into the tube and push down moderately firmly to ensure that the pellet has been lodged into the flesh of the fish without crushing the pellet.

5. Apply light pressure to the incision and remove the rod. To prevent possible infection from the incision, place a small amount of Betadine and mucus over the incision.

6.3.4 Assessment of reproductive development stage

The process of cannulation is performed on fish to determine the stage of developing oocytes (ie. whether they have completed vitellogenesis and hence would respond to hormone induction methods). To interpret oocyte samples (after cannulation) see written and visual descriptions of both macroscopic and microscopic oocytes in Section 5.1.1.

The process of cannulation

An endometrial tube is inserted into the gonadal pore, just behind the anal opening (Figure 6-6).

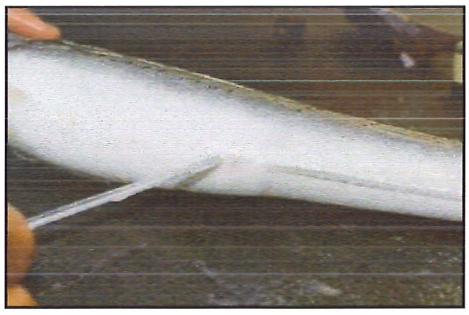


Figure 6 - 6 Locating the gonadal pore

To ensure that the tube is inserted into the correct opening, it is best to angle the tube perpendicular to the fish, with the tube pointing slightly towards the back of the fish (Figure 6-7).

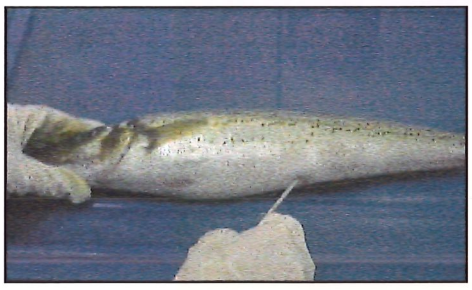


Figure 6 - 7 Inserting the endometrial tube into the gonadal pore

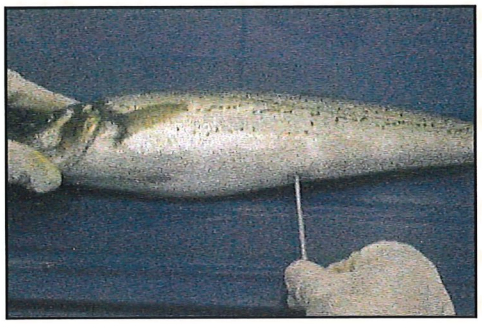


Figure 6 - 8 Holding the tube perpendicular to the fish while continuing to insert the endometrial tube

Once the tip of the endometrial tube has been inserted into the gonadal pore, angle the tube perpendicular to the fish while applying a small amount of pressure to ensure that the tube is inside the gonadal pore (Figure 6-8).

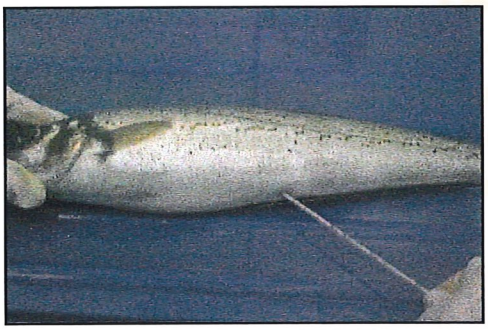


Figure 6 - 9 Collecting the oocyte sample

Angle the tube towards the head of the fish and insert the tube approximately 2-3cm, carefully and gently rolling the tube as you go.
Once the tube is inserted, pull back the inside of the tube approximately 1–2cm (Figure 6-9).

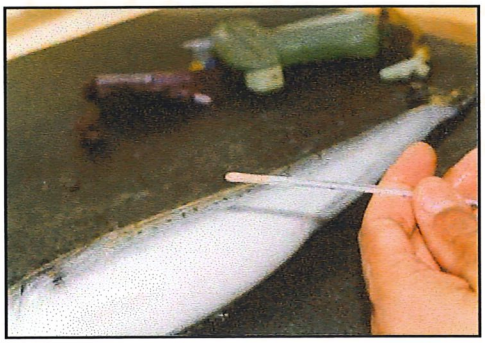


Figure 6 - 10 Assessing the oocyte sample

Rotate and gently remove. If there is any resistance, push the inner tube back in a small amount until no resistance is felt. Assess the sample macroscopically or collect sample for examination under a microscope (Figure 6-10).

Visual assessment of females in tanks

A visual assessment of the reproductive stage of KGW female can be obtained by examining fish in tanks. For convenience, we have defined 3 development stages.

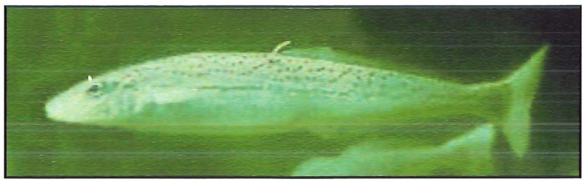


Figure 6 - 11 Visual assessment of the reproductive development of a KGW female (stage 1)

Stage 1

No visible development in the dorsal side (belly) of the fish. At this stage oocytes can range from immature to advanced yolked. Oocytes have not begun the final stages of maturation (Figure 6-11).

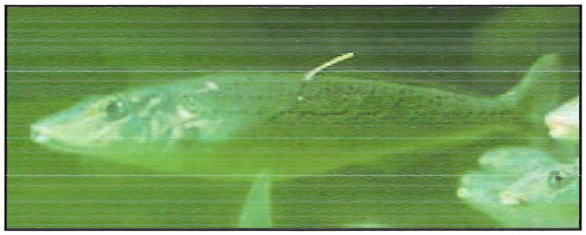


Figure 6 - 12 Visual assessment of the reproductive development of a KGW female (stage 2)

Stage 2

The dorsal side of the fish becomes more prominent, developing a 'fatty' appearance. The final stages of maturation have begun (Figure 6-13).

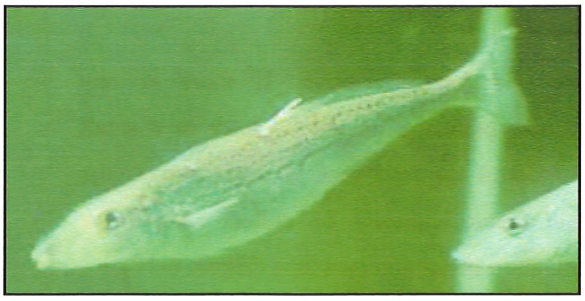


Figure 6 - 13 Visual assessment of the reproductive development of a KGW female (stage 3)

Stage 3

A definite step exists between the 'belly' and the gonadal pore. A batch of eggs is fully hydrated and is ready for 'tank' spawning or to be manually stripped.

6.3.5 Egg collection

There are 2 methods of egg collection:

- collection of eggs via outflow from broodstock tank after voluntary spawning, and
- manual stripping of eggs and milt from broodstock for artificial fertilisation.

Egg collection from tanks

Like most marine finfish eggs, KGW eggs are positively buoyant, floating to the surface of the tank. Eggs are thus continuously flushed via the outflow of the broodstock tank to an egg collector on the outside of the tank.

Figure 6 - 14 Egg collector attached to the side of the broodstock tank

The egg collector consists of 500µm nylon mesh sewn into a basket, supported by a ring of polypipe at the top and a ring of weighted polypipe on the bottom. The mesh bag is suspended in a round 110L tub just slightly larger than the collector, with an 50mm outlet (Figure 6 - 14).

Procedure for collecting eggs

- Switch the tank to bottom outflow, the morning following spawning.
- Leave the collector for approximately 15 mins., allowing viable (fertilised) eggs to float to the surface and unfertilised or dead eggs to sink to the bottom.
- Collect viable eggs with a bowl, gently skimming the surface of the water, and put in a 20L bucket with a small amount of water from the broodstock tank.

- Leave the collector static for another 10–15 mins. after collecting the majority of eggs, allowing additional viable eggs to float to the surface.
- Collect remaining eggs and fill the bucket to 20L with water from the broodstock tank in preparation for counting (Section 6.3.6).
- Collect non-fertile or dead eggs by lifting the screen out and washing the remaining eggs into another 20L bucket for subsequent counting.

Artificial fertilisation (manual egg collection)

Artificial fertilisation of fish is not performed regularly at SARDI as it is labour-intensive and can result in lower quality eggs than natural or voluntary tank spawning. However, it is sometimes necessary, with fish that undergo the final stages of maturation (usually after hormone induction) but do not spawn in captivity. This technique is being used at SARDI to synchronise supply of larger numbers of eggs than that available from broodstock left to spawn spontaneously. Hormone injection of a set number of broodstock is followed by manual stripping to supply millions of eggs at one time rather than a hundred thousand produced erratically with spontaneous tank spawning. This procedure should allow controlled supply of large eggs required to stock commercial scale larval rearing tanks (5-20kL). Artificial fertilisation can be performed only when female oocytes have undergone the final stages of maturation, hydration, and are ready to be released. It should be performed in an area free of sunlight with a temperature similar to the broodstock tank.

At SARDI, the dry method of fertilisation is used as outlined in Tucker (1998).

Proceedure for manual stripping

- Capture fish (Section 4.2.1).
- Anaesthetise fish (Section 4.3).
- Once fish are fully anaesthetised, hold the fish dorsal side up (ie. belly up) with one hand cupped around the snout and supporting the back of the fish, while holding it close to your body. Gently place a paper towel along the belly of the fish to remove excess water (Note: do not rub the surface of the fish).
- Males: with the other hand, starting from one-third from the head of the fish and moving towards the anus, press 2 fingers together to form a small 'V' with the tips of the fingers, with moderate pressure along the mid line of the belly. Place a hypodermic syringe (without a needle attached) on top of the gonadal pore (it is usually best if someone else assists with

this) taking up the milt once it is released (Figure 6-15). Store milt in syringes or airtight containers until required.

- Females: to strip the eggs from females, turn the fish up the right way holding the head closest to your body. Using the other hand, starting from one-third from the head of the fish and moving towards the anus, press 2 fingers together to form a small 'V' with the tips of the fingers, with moderate pressure along the mid line of the belly (Figure 6-16). If the oocytes are hydrated, eggs will be released once minimal pressure is placed on the abdomen of the female. Drain eggs directly into a clean, dry bowl. Avoid contaminating the bowl with seawater.
- Distribute milt over eggs and mix using a previously sterilised feather. This allows the eggs to be completely coated by the milt, with minimal harm. At this stage, seawater can be added to stimulate the sperm and fertilisation will occur.
- Once eggs are fertilised, they should be left for a couple of hours, allowing the chorion to harden and initial development to take place. After this period they can be handled gently for counting and assessment of egg quality.

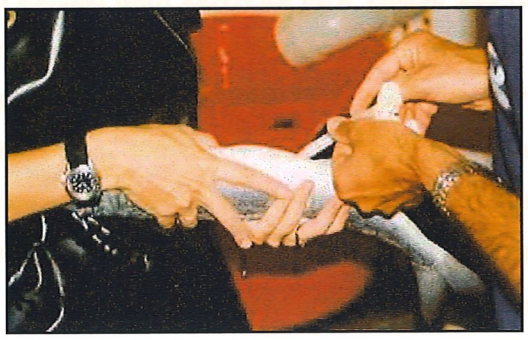


Figure 6 - 15 Collection of milt using a syringe from a male KGW

Figure 6 - 16 Stripping of hydrated eggs from a ripe female

6.3.6 Egg counts

Volumetric method

- Gently agitate eggs in a 20L bucket using aeration.
- Take a sample using a 10mL pipette and place in an egg-counting tray (plastic block with 'v' shaped wedges cut into the block in lines so that eggs can rest in the trough of the 'v' and be easily counted). Take the mean of at least 3 counts.

- Check the quality of the eggs under a microscope (Section 6.3.7). Poor quality eggs should be not be stocked into systems.

To calculate the total number of eggs:

- The mean number of eggs in 10mL x 100 x volume of bucket (L) = total number of eggs.
- Eggs can then be stocked into an incubator

6.3.7 Egg quality

Egg quality, defined as those characteristics of the egg which determine its capacity to survive, should be assessed before stocking to ensure optimum survival of larvae. There is little agreement regarding reliable methods for the assessment of egg quality (Bromage and Roberts 1995). At SAASC, the fertilisation rate, physical appearance of the egg, hatch rates and larval survival are used as indicators.

The percentage of eggs fertilised is a good indicator of egg quality with survivors from batches of eggs with poor fertilisation rates generally performing badly at all subsequent stages of development (Springate *et al* 1984; Bromage and Cumaranatunga 1988; Bromage *et al* 1992 as cited in Bromage and Roberts 1995). However, unless individual spawners can be identified, this is not an absolute indicator. To determine the fertilisation rate, eggs should be examined under a microscope to check that they are developing (Section 5.1.2). The percentage fertilisation is calculated (see below) by the number of fertile eggs divided by the total number of eggs (buoyant and non-buoyant).

The physical appearance of the egg should also be considered. The appearance of the chorion, the shape of the egg, its transparency and the distribution of oil globules can be related to egg quality (Figures 6 - 17 and 6 - 18) (Kjørsvik *et al* 1990). Good quality KGW eggs should be spherical in shape (0.85–0.94mm in diameter), transparent with one oil droplet (0.25–0.26mm in diameter) and have symmetrical cleavages (Figures 6 - 19 and 6 - 20).

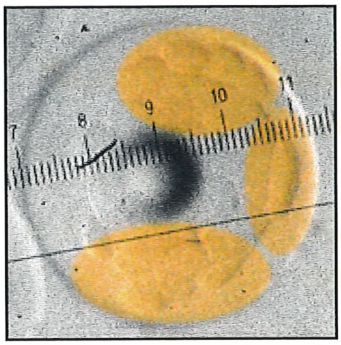


Figure 6 - 17 Poor quality KGW egg (16 cell stage) where cell size and distribution is uneven and cells are bulging to the top and bottom rather than forming a circular shape

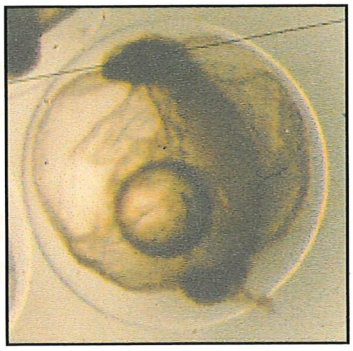


Figure 6 - 18 Poor quality KGW egg (stage 12) where the yolk sac is opaque in colour and appears to be shrivelling up

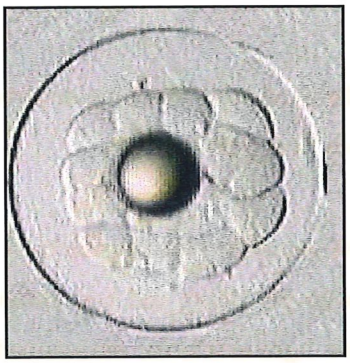


Figure 6 - 19 Good quality KGW egg (16 cell stage) and the cells are an even size and distributed evenly around the oil droplet in a circular pattern

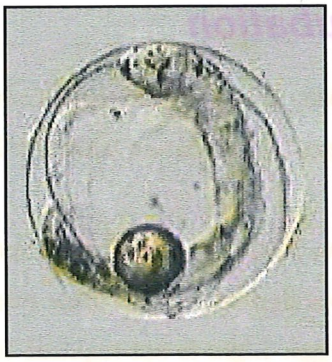


Figure 6 - 20 Good quality KGW egg (stage 12) where the embryo is developing well and the yolk sac is clear and full

Once incubated, hatch rates and early larval survival should be assessed before stocking into larval rearing tanks. Good quality eggs have enough endogenous reserves to sustain the growth and development of larvae to first feeding. If larvae suffer high mortality during early development they should not be stocked into rearing tanks.

Calculations

Fertilisation rate = $\frac{\text{Number of fertilised/developing eggs x 100}}{\text{Total number of eggs}}$

Hatch rate = $\frac{\text{Number of larvae hatched x 100}}{\text{Total number of eggs stocked}}$

Larval survival = $\frac{\text{Number of surviving larvae x 100}}{\text{Total number of larvae stocked}}$

6.4 Incubation

Using a beaker, gently transfer eggs from the bucket into the incubator. Stocking density is generally 500-1000 eggs/L. Incubation tanks are 200L conical bottom fibreglass tanks.

Outlet screens made of 300µm-nylon screen are filled to the overflow pipe (Figure 6-21). Incubation tanks are provided with a low flow rate (40-100% exchange per hour) and low aeration to gently agitate the eggs. Light is not required for hatching and low light levels are maintained to optimise yolk sac absorption.

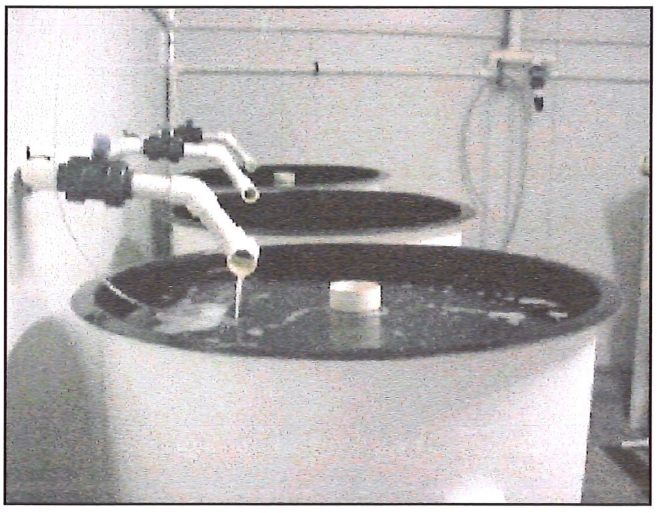


Figure 6 - 21 Egg incubation tanks at SAASC

6.5 Hatching

6.5.1 Time to hatch

The time taken for eggs to hatch is variable depending on water temperature. Hatch times within the optimal temperature range for incubation of KGW are shown in Table 6 - 3.

Table 6 - 3 Time taken for KGW eggs to hatch at 16°C, 19°C and 22°C

Temperature (°C)	Time to hatch (hr)
16	77
19	48
22	39

6.5.2 Hatching rate

Incubation studies on KGW eggs showed that the combined effects of temperature and salinity clearly affected the hatch rate of KGW eggs. Temperature was found to be the dominant environmental parameter affecting embryonic development, with temperatures >24°C causing total mortality. Salinity had a less marked effect although low salinities (<30ppt) caused high mortality, possibly due to complications with negatively buoyant eggs.

The optimum hatching rate of KGW eggs was achieved when the following conditions were maintained:

- salinity: >35ppt and <45 ppt, and
- temperature: 16–19°C.

6.5.3 Calculating hatch rate

Hatch rates should be calculated to assess the quality of larvae and to calculate the total numbers of larvae for stocking purposes.

The volumetric method is the quickest and most convenient method.

- Using a 10ml pipette, take a sample from the incubation tank (gently agitating the water) and place in a Petri dish.

- Count the number of larvae under a microscope or by illuminating the Petri dish from below using a torch.
- Take the mean of at least 3 counts.

Based on the number of larvae in a 10ml sample, estimate the total number in the tank as follows:

Number of larvae in 10ml x 100 x volume of tank (L) = total number of larvae in tank.

6.5.4 Transfer to tanks

Larvae can be transferred into larval rearing tanks before completion of yolk sac absorption and eye and jaw development (ie generally 1-2 days prior to first feeding, 4 days post hatch at 19°C).

Larvae can be condensed through a submerged 300µm screen and gently ladled directly into larval rearing tanks. Larvae should not undergo any extreme changes in temperature or salinity.

6.5.5 Transfer to other places

Larvae can be transferred to other locations via sealed plastic bags housed in polystyrene boxes.

- Half fill a double sealed bag with water from the incubation tank and aerate (ie. to maintain constant water temperature and salinity).
- Larvae from the tank are condensed by pouring through a submerged 500µm screen.
- Larvae are gently ladled into the bag once condensed.
- Once a sufficient number of larvae have been added to the bag, remove air from the bag and replace with oxygen via a compressed oxygen tank (fill bag to capacity with oxygen).
- Secure the opening of the bag with two rubber bands.
- Place the bag (containing larvae) inside an additional bag and double seal with rubber bands.
- Gently place the bag inside a polystyrene box.
- Add 'stuffing' if required to reduce any movement of bags within the box.

Chapter 7: Larval Rearing

7.1 Larval Biology

The larval stage broadly covers hatching of the embryo through to transformation of the larvae into a juvenile (Bond 1979). This period can be further defined into yolk sac stage, pre flexion, flexion and post flexion (Neria *et al* 1998).

Like most marine teleosts, KGW larvae hatch at a small size (2.4–2.6mm total length) with no functional gut, eyes or mouth. After hatching, larvae go through a series of physical and behavioural stages of development. Transformation into a juvenile is completed when a larvae takes on the characteristics of a adult, such as developed fins, rays, bones, and scales and have an improved ability to feed (Tucker 1998). In the wild, KGW have an extended larval period lasting for about 60–80 days (Bruce 1989 as cited in Kailola *et al* 1993). The larval period can be shortened in reared KGW larvae depending on the temperature and feeding regime during culture (about 50 days at 20–22°C temperatures). A summary of major developmental events occurring over the first 12 days of KGW development is presented in Figure 7 - 1.

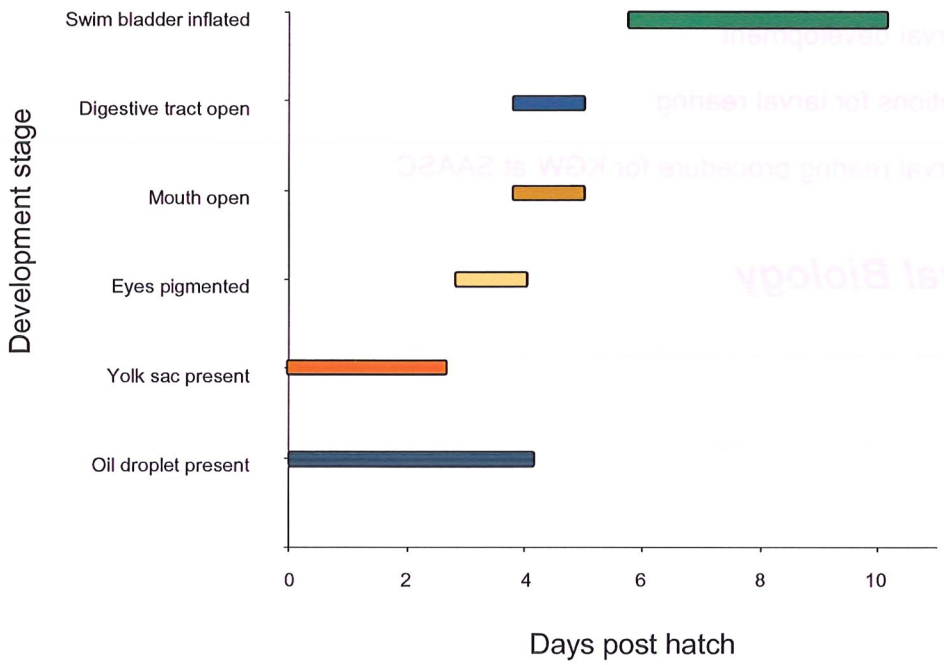


Figure 7 - 1 Overview of KGW early larval development stages for KGW larvae cultured at 20°C

7.2 Larval development

7.2.1 Yolk sac stage (1–3 days post hatch (dph); 2.4–2.9mm)

As the name suggests, this stage (Figure 7 - 2; 7 - 3; 7 - 4 and 7 - 5) is characterised by the presence of the yolk sac (sac-like extension of the embryonic gut) containing the yolk, the nutritive material for embryos and newly hatched larvae, and a single oil droplet. At this stage larvae lack the ability to feed externally and all nutritional requirements are supplied from the yolk and oil droplet. Larvae in the yolk sac stage move passively near the water surface. Hatched larvae are only lightly pigmented.

7.2.2 Approaching pre flexion (4 dph; 2.9-3.0mm)

As larvae approach pre flexion, their eyes become fully pigmented and their mouths and gut (straight at this stage of development) open (Figure 7 - 6). The yolk sac is mostly absorbed and there is only a small remnant of oil droplet.

7.2.3 Pre flexion (4–18 dph; 3.0–5.6mm)

Pre flexion is marked by the onset of exogenous feeding as the eyes, mouth and digestive system become functional (Figure 7 - 7). At this stage the swim bladder begins to form (6 dph) and inflate (8–10 dph) (Figure 7 - 8). The gut continues to develop, but remains straight at this stage. The notochord is straight. Larvae begin actively swimming as they search for food. During pre flexion single melanophores develop in a series along the mid-line trunk and tail. Internal and external pigmentation begins to develop, visible near the hindbrain and dorsally over the swim bladder and gut (Neria *et al*, 1998).

7.2.4 Flexion (22–29 dph; 5.6–6.3mm)

In flexion larvae, the notochord is turning upwards and the caudal skeleton is forming (Tucker 1998) (Figure 7 - 12). Most dorsal melanophores disappear by the end of flexion stage, leaving only a few along the trunk and tail (Neria *et al*, 1998). At this stage larvae start to move down through the water column but still remain in the top half of the tank, often feeding off the sides.

7.2.5 Post flexion (29–50 dph; 6.3mm–14.10mm)

In post flexion, the notochord has reached its final position and the caudal skeleton is mostly formed (Tucker 1998) (Figure 7 - 13 and 7 - 14). Dorsal pigmentation on the trunk and tail reappears during the post flexion stage as discrete pigment blotches, each comprising of 3-4 pairs of stellate melanophores (Neria *et al* 1998). Post flexion larvae begin to settle and feed in the lower half of the water column.

7.2.6 KGW larval development series

Larvae in this series were cultured at 20°C.

YS=Yolk sac; OD-Oil droplet; TL= Total length

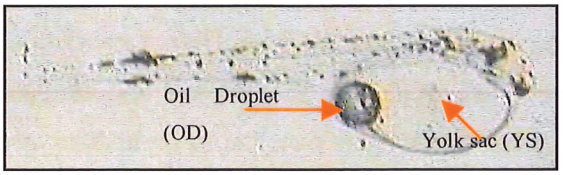


Figure 7 - 2 KGW larvae (0 dph)

Yolk sac stage

0 dph (hatch)

Feeding off yolk sac, oil droplet visible. TL = 2.4-2.6mm; YS = 0.9 x 0.5mm; OD = 0.2mm.

Figure 7 - 3 KGW larvae (1 dph)

1 dph

Feeding off yolk sac, oil droplet still visible. TL = 2.4-2.6mm; YS = 0.6 x 0.3mm; OD = 0.14mm.

Figure 7 - 4 KGW larvae (2dph)

2 dph

Feeding off yolk sac, oil droplet still visible. TL = 2.6-2.8mm; YS = 0.4x0.3mm; OD = 0.13mm.

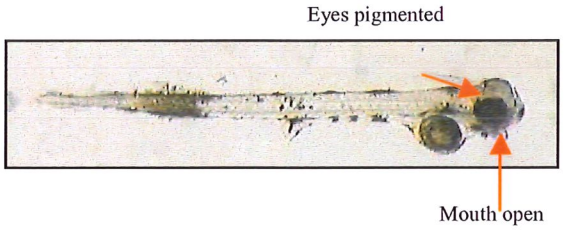


Figure 7 - 5 KGW larvae (3 dph)

3 dph

A small amount of yolk sac and oil droplet present. Eyes starting to pigment and mouth open.

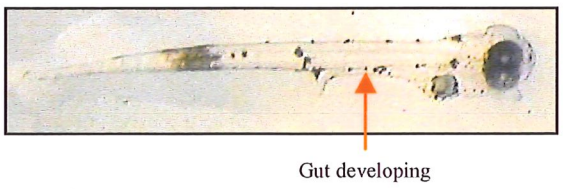


Figure 7 - 6 KGW larvae (4 dph)

Approaching pre flexion

4 dph

Eyes fully pigmented, gut developing and mouth open. A small amount of yolk sac and oil droplet remains.

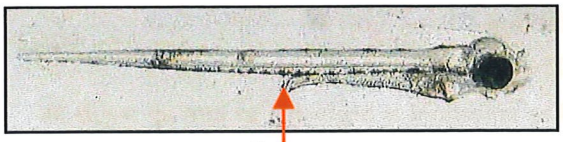


Figure 7 - 7 KGW larvae (6 dph)

Pre flexion

6 dph

Oil droplet completely absorbed. Gut opening. Larvae begin to feed. TL = 3.2mm.

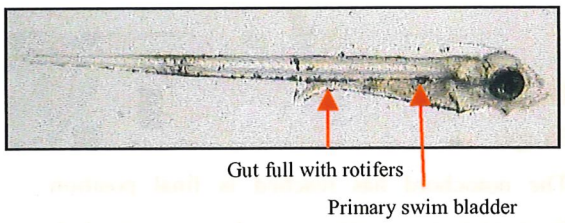


Figure 7 - 8 KGW larvae (7 dph)

7 dph

Gut open, full of rotifers and continuing to develop. Primary swim bladder visible. TL = 3.6mm.

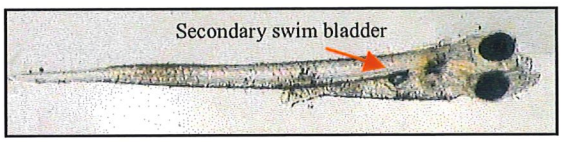


Figure 7 - 9 KGW larvae (8 dph)

Primary inflation

8 dph

Swim bladder visible, continuing to develop. TL = 3.4-3.6mm.

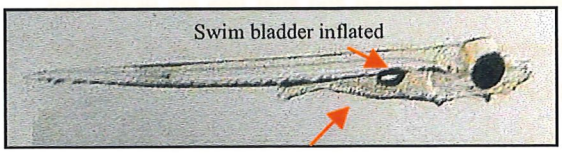


Figure 7 - 10 KGW larvae (10 dph)

10 dph

Swim bladder inflated. TL = 4.5.

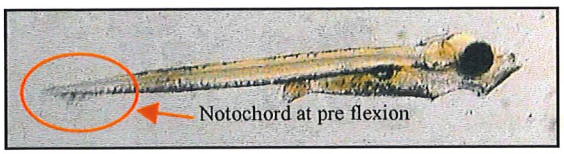


Figure 7 - 11 KGW larvae (18 dph)

Pre flexion

18 dph

Increased pigmentation becomes visible. TL = 5.3mm

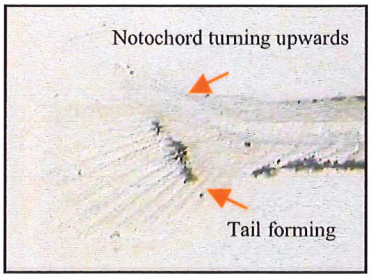


Figure 7 - 12 Tail of KGW larvae during notochord flexion (22dph)

Flexion

22 dph

The notochord is beginning to turn up wards as the caudal skeleton is forming. TL = 5.8mm.

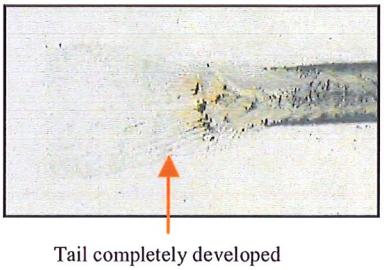


Figure 7 - 13 Tail of KGW larvae after notochord flexion (36 dph)

Post-Flexion

36 dph

The notochord has reached is final position. Pigmentation continues and is particularly evident on the truck and tail.

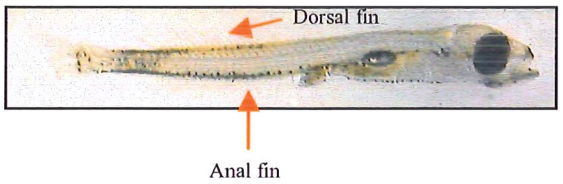


Figure 7 - 14 KGW larvae (36 dph)

36 dph

At this stage the caudal skeleton is mostly formed. The dorsal and anal fins are developed. Pigmentation continues becoming more evident. TL= 8.0mm

7.2.7 Examination of larvae

It is essential to monitor growth and development of larvae for effective larval culture. Ten larvae should be regularly removed from tanks and viewed under a microscope to record growth data, developmental stages and gut contents. To view larvae, place larvae on a Petri dish. Remove excess water using a pipette. Leave the larvae lying sideways in a small amount of water. View larvae under a dissecting microscope with a transmitting light base and ocular micrometer fitted to eyepiece.

7.3 Options for larval rearing

In finfish larval rearing, 2 main techniques have been established: intensive and extensive. Our research has focused on intensive larval culture, which involves producing high densities of larvae in flow-through or recirculated seawater tanks under controlled conditions. There are different degrees of intensive culture based on stocking densities, which can vary from 30 larvae/L to 200 larvae/L. In intensive systems feeding (ie. type, quantity and quality), temperature, salinity, aeration, lighting and water quality are controlled. Feeding protocols allow the nutritional status of larvae to be monitored and adjusted if necessary. This can be done by altering the size and type of live and inert feeds offered to larvae and nutrition provided by these feeds. Labour and capital costs of intensive larval rearing are high. Larval rearing is the most technically demanding period of KGW culture.

In extensive salt water systems, ponds are stocked with eggs or larvae, which grow using zooplankton and phytoplankton as a food source. Blooms of phytoplankton and zooplankton in ponds can be managed through nutrient enrichment and water exchanges. There is little to no control over environmental conditions or food production within the pond, and this can result in variable survival of larvae. The labour and capital costs of extensive rearing are low. Extensive pond culture for KGW has been investigated by a Playford Trust Memorial Scholarship PhD student, University of Adelaide. This study mostly concentrated on phytoplankton and zooplankton dynamics, which precede stocking of eggs and larvae. Table 7 - 1 provides a comparative overview of intensive and extensive techniques.

Table 7 - 1 Comparison of intensive and extensive larval rearing techniques

	Intensive	**Extensive**
Labour	High	Low
Management	High	Low
Cost	High	Low
Area	Low	High
Capital costs	High	Low
Technical expertise	High	Low
Stocking density	High	Low
Water exchange	High	Low
Environmental control	High	Low
Natural productivity	Low/ Nil	High
Supplementary feeding	High	Low
Disease risks	High	Low

7.4 Larval rearing procedure for KGW at SAASC

Two main phases are involved in larval rearing of KGW at SAASC:

- green water phase (until the completion of rotifer feeding), and
- clear water phase (from the start of *Artemia* feeding).

The procedure developed at SAASC is based on literature reviews, observation and discussions with operators of commercial marine finfish hatcheries (2–30 million fingerlings per annum) in Italy and Greece. The procedure is considered to follow current world best practice for commercial finfish fingerling production, with minor changes to accommodate the timing of the developmental stages of KGW. It should be noted that considerable innovation and product development occurs continuously in marine finfish production such that methods used are constantly evolving.

A summary of operational procedures followed at SAASC for KGW larval rearing is provided in Figure 7 - 15.

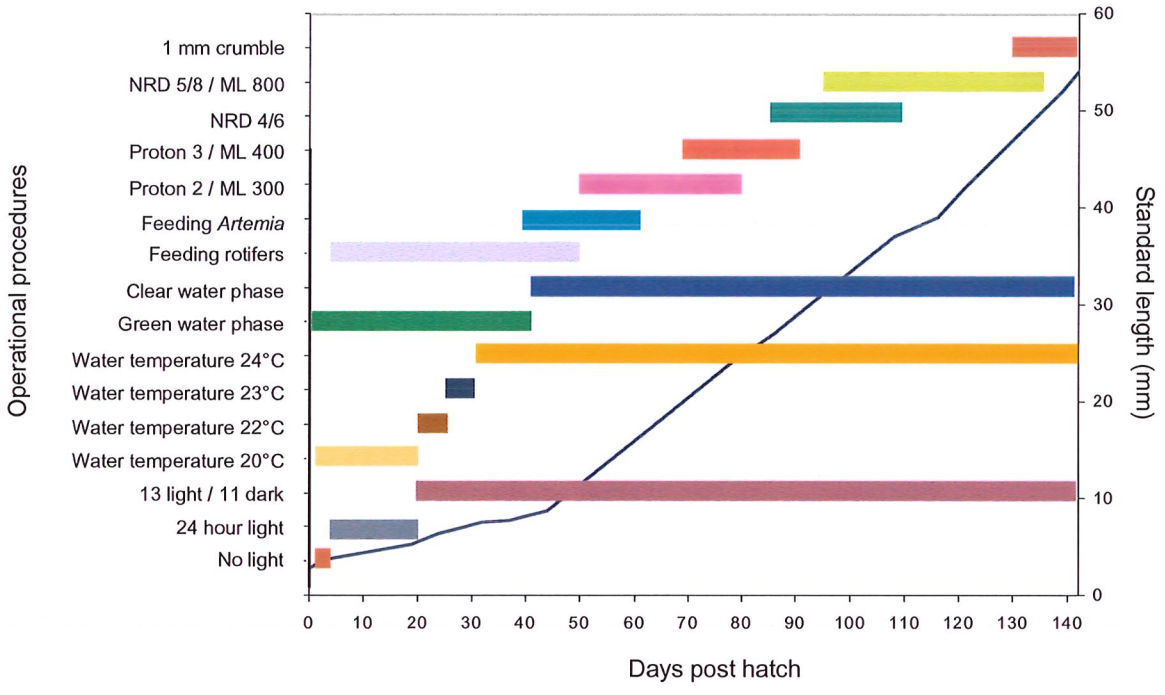


Figure 7 - 15 Overview of operational procedures for the larval rearing of KGW and growth (standard length) of KGW larvae over time (dph)

Green water phase

The 'green water' phase in initiated at the beginning of larval rearing and is characterised by the maintenance of micro-algae in larval rearing tanks. Larval rearing tanks are seeded with micro-algae before stocking with newly hatched larvae to stabilise water, buffering chemical changes and to maintain the nutrition of rotifers in the tank. Rotifers are the first external feed offered to larvae. Enriched rotifers are fed to larvae as determined by rate of consumption.

Micro-algae

The 'green water' phase used at SAASC is in reality a 'brown water' phase as *Isochrysis galbana.* Tahitian strain (T. Iso) is the micro-algae species added daily to larval rearing tanks. T. Iso is the species of choice because it can be cultured to good cell densities (5 million cells/mL) in mass culture (open FRP tanks) and provides good levels of highly unsaturated fatty acids (HUFAs) essential for larval development. Cell densities in larval rearing tanks are maintained around 400,000 cells/mL. As cell density counts can be time-consuming, algal density can be estimated by eye, adding algae slowly until the bottom of the tank is just visible.

Rotifers

Before rotifers are added to larval rearing tanks, they are enriched to maximise their nutritional profile, improving the growth and survival of larvae. Rotifers are enriched with high nutritional quality micro-algae (*Pavlova lutheri* or T. Iso) for 18–24 hours before feeding to larvae. Currently enrichment products for rotifers are not used at SAASC as these have tended to clump rotifers causing loss of feed to larvae. Ongoing product development has improved performance of these products which combined with refinement of enrichment tank operations is expected to allow application of these products in our research in future. It is important that rotifers are rinsed well after any enrichment procedure by maintaining concentrated rotifers in a harvester and flushing with seawater for 5–10 minutes or until the water is clear.

KGW cultured at 18–20°C are ready to begin feeding at approximately 5–7 days post hatch (dph). At this stage, KGW larvae have small mouths (about 0.56mm mouth gape), a short digestive tract and few functional enzymes, which limit the type of first feeds suitable. Research has indicated that small strain rotifers (S-type), *Brachionus rotundiformis,* is an appropriate first feed for KGW larvae. S-type rotifers are added to tanks from 4 dph to promote first feeding of larvae. It is important to add rotifers before larvae are ready to begin feeding as there is a narrow window where larvae start feeding. If food is not available when larvae are ready to begin feeding, they will become too weak to strike and ingest food and will ultimately die if the 'point of no return' is exceeded.

Enriched S-type rotifers are added each morning to larval rearing tanks for 7–10 days. After this, enriched large strain rotifers (L-type), *Brachionus plicatilis,* are added to tanks and the use of S-type rotifers stops after a 5-day overlap. Rotifer densities should not be less than 5/mL or exceed 20/mL for an extended period of time throughout the green water phase. The mean of a minimum of 3 counts of rotifers in larval rearing tanks (for rotifers counts *see* Section 10.2.4) should be taken at least each morning to estimate densities and feed requirements.

If larvae are consuming only small amounts of rotifers, they can be maintained in larval rearing tanks with regular additions of micro-algae to maintain nutrition and density (ie. through reproduction of rotifers within the tank).

Copeopds

Copepods are not currently being used as a first feed for KGW at SAASC. The nutritional profile, size and movement is such that they could be considered as an alternative or supplementary first feed for larval KGW with possibilities of enhancing larval survival.

Tanks

Larval rearing at SAASC is done in 1.8kL circular FRP tanks with a conical base (10°) (Figure 7 - 16). Tanks have dark blue sides (enabling prey to be easily detected by fish) with a central 400mm diameter white circle at the bottom (allowing mortality, water quality and density of micro-algae to be easily assessed by eye). Inlet pipes are at the top of the tank and water is removed through a central standpipe fitted with an appropriate size of mesh screen (Figure 7 - 17). Water level in tanks is set using a moveable external standpipe.

Figure 7 - 16 Larval rearing tanks at SAASC

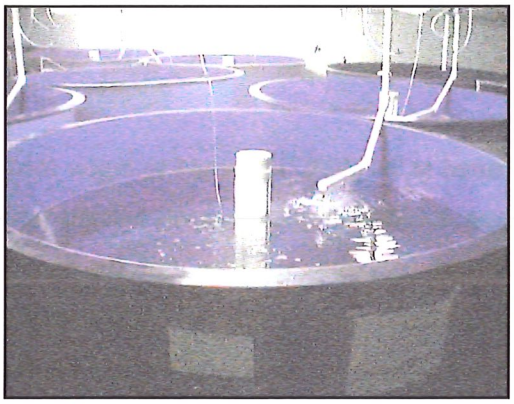


Figure 7 - 17 Top view of larval rearing tanks at SAASC

Screens

60µm screens are used to retain rotifers and allow water to flush out during green water culture (Figure 7 - 18). A 150µm screen can be used if rotifer numbers are high or they need to be removed.

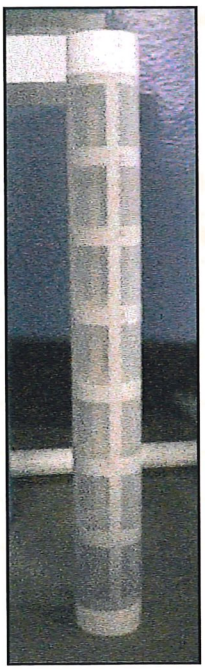


Figure 7 - 18 Central standpipe fitted with a 60µm screen

Water flow rate

A low flow rate (5–10% of the tank volume/hr) is used to increase water quality throughout the green water phase. At latter stages of this phase water flow is increased (10-20% tank volume/hr) in combination with twice-daily additions of micro-algae.

Lighting

No light is required up to 4 dph. Continuous (24 hour) light is provided between 4–50 dph to promote feeding and maintain growth. Illumination is supplied through two standard 36 watt cool white fluorescent tubes mounted in water resistant (1P 56 rated fittings) suspended 50–70cm above each tank. A dark interval is incorporated (16 hour light: 8 hour dark) when flow rates are high and feed cannot be provided over a 24-hour period.

Water temperature

Water temperature is a prime determinant of growth rate for KGW larvae. Temperature tolerance and optima may change as larvae develop. Trials conducted at SARDI have shown that eggs and newly hatched larvae prefer temperatures between 16–19°C, while more advanced larvae show improved growth at higher water temperatures after their tolerance to these has developed. To promote maximum growth and survival, the water temperature is increased through the larval rearing period as described in Table 7 - 2.

Table 7 - 2 Temperature regime used during larval rearing at SAASC

Days post hatch (dph)	**Water temperature (°C)**
0-5	19
6-20	20
21-26	22
27-33	23
34-140	24
140+	25

Surface Skimmer

For KGW, swim bladder inflation occurs between 6–10 dph. Swim bladder inflation is an important stage of development for marine finfish larvae as it provides buoyancy, which reduces energy expenditure, required to maintain position in the water column. Generally swim bladder inflation coincides with complete or partial exhaustion of the yolk sac and oil droplet following the beginning of first feeding (Doroshev *et al* 1981; Chatain 1989; Soares *et al* 1994). In early marine finfish culture operations, rates of normal swim bladder were low, commonly varying between 0–25% (Chatain 1989; Chatain and Ounais-Guschemann 1990; Chatain and Corroa 1992). The absence of functional swim bladders in hatchery-reared fish can result in decreased growth (Chatain 1989), skeletal malformations (Paperna 1978; Kitajima *et al* 1981; Chatain 1989; Henderson-Arzapalo *et al* 1992) and high mortality of fish exposed to stress (Chatain 1989).

In marine finfish hatcheries, swimbladder inflation is maximised using floating surface skimmers (Figures 7 - 19 and 7 - 20) which use an air curtain blown at an angle to the water surface to constantly concentrate oil films in larval rearing tanks. Oil films are an unavoidable result of the breakdown of the algae and enriched feeds added to tanks. To maintain an oil-free surface, it is

necessary to frequently clean the surface skimmers (at least twice a day), by blotting up the concentrated contents in the skimmer with absorbent sheet or scooping with a small container.

Maintaining a water surface free of oil films allows larvae to access the surface, take a gulp of air and initially inflate their swim bladders. This is important as the larval physoclistous swim bladder has a temporary pneumatic duct, which provides a passage for gas transfer from the digestive tract to allow initial inflation (Steen 1970; Doroshev *et al* 1981). This pneumatic duct eventually degenerates (Doroshev *et al* 1981) and if inflation fails swimbladder development stops and the organ is not functional (Chatain 1989). Following successful inflation, the proximal part of the pneumatic duct degenerates and its distal part develops into the gas gland, which subsequently controls gas deposition in the swim bladder (Steen 1970).

During larval culture, skimmers must be cleaned as many times as possible each day (at least 4 times a day) using paper towel to absorb surface debris inside the collection area.

Swimbladder inflation is observed as a two-stage process. Initial or primary inflation (Figure 7 - 8) is characterised by the appearance of a light refractive bubble in the primordial swimbladder. This occurs between 6–8 dph with KGW. Following this is an expansion stage (Chatain 1989) which occurs between 8–10 dph with KGW (Figure 7 - 9).

Surface skimmers can be constructed from a variety of materials such as PVC piping (Figure 7 - 19), or polystyrene foam sheeting which can be cut (ie. hot wire) to a template provided to suppliers (Figure 7 - 20).

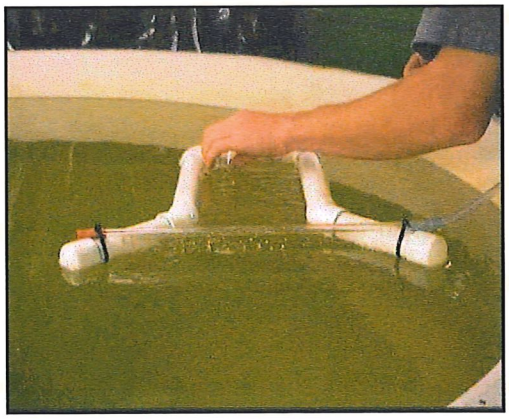


Figure 7 - 19 PVC surface skimmer

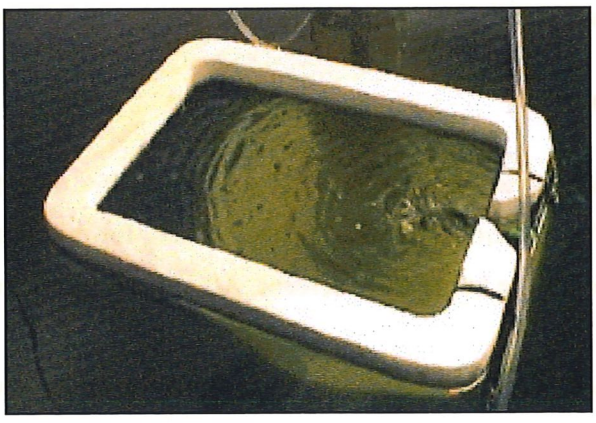


Figure 7 - 20 Polystyrene foam surface skimmer

Growth rates

KGW have a long larval phase (about 2–3 months), with slow growth. After this, growth rates increase substantially. Elevated temperature within the ecological limits of the species, feeding to excess and maintaining high water quality, will maximise growth rates and substantially reduce the length of time of the larval phase.

Cleaning

During the first 14 days of green water culture, minimal cleaning is done apart from spot siphoning of accumulated debris. If "red-pink" bacteria colonies are observed on the base of the tank, these are removed, as they have been linked with high mortality. Outlet screens should be washed at least once a day. Bottom valves should be opened daily for 5–10 seconds to remove any debris that may have settled and become anoxic.

7.4.2 Clear water phase

Algae

Algae is not added during the clear water phase as flow rates become too high for it to persist in the larval rearing tanks and it is assumed that *Artemia* added will be consumed before they derive any background benefit from microalgae.

Screens

The size of the screen is progressively upgraded as needed, starting from 150µm during the clear water phase. Once weaning begins, screen size is increased to 500µm and finally 2mm.

Rotifers

Once larvae begin feeding on *Artemia*, rotifer density in larval rearing tanks can be reduced to 5–10/mL. Rotifers are added each morning for the first 5 days of the clear water phase, as they flush through the larger mesh screens. After this time rotifer co-feeding is stopped.

Once larvae are completely weaned off rotifers (approximately 50 dph), the photoperiod is changed to 16 hours light: 8 dark.

Artemia

Artemia nauplii are added to tanks when larvae are about 10mm long (40 dph for larvae cultured at 20°C). Enriched stage II (2 dph) *Artemia* nauplii are fed to larvae in the morning and evening. Initially, rotifer co-feeding occurs and *Artemia* are added at a density of 0.5/mL. Once the majority of larvae are feeding on *Artemia*, the density can be increased to 1/mL. Before reducing the number of rotifers added to culture tanks it is important to establish that >70–80% of larvae are feeding on *Artemia*.

To check whether larvae are feeding on *Artemia,* about 5–10 larvae (depending on time constraints) are examined under a microscope. Those feeding on *Artemia* will have a visibly orange gut.

Calculation of the number of *Artemia* nauplii to feed is based on the hatching efficiency of the cysts used. The yield of nauplii can vary from 200,000 to 320,000 cysts in 1g of premium-grade *Artemia* cysts (premium-grade cysts have a greater than 85% hatch rate).

Due to the variable quality and supply of *Artemia* cysts it is advisable to measure the hatching efficiency (number of nauplii hatched per gram of cysts product) under the hatching conditions used. *Artemia* feeds can then be based on this figure and adjusted according to consumption rate (demand) of growing larvae (time taken for *Artemia* to be cleaned from the tank by larvae).

Weaning

Weaning begins at about 65 dph (Figure 7 - 15). Larvae should be weaned by 80 dph at which stage *Artemia* co-feeding stops. This regime is conservative and future research will concentrate on shortening the *Artemia* feeding phase to reduce production costs. The feeding schedule must correspond to the light schedule to ensure that larvae are not wasting energy looking for food that is not there. Nippai feeds (ML 300 (300µm), ML 400 (400µm) and ML 800 (800µm)) or INVE feeds (Proton 2 (150-300µm), Proton 3 (200-400µm), NRD 4/6 (400-600µm), NRD 5/8 (500-800µm)) are used at SAASC as the initial weaning diet after which Pivot (now Skretting Australia) 1mm crumble and 1.5mm Barramundi crumble are fed for further ongrowing.

Table 7 - 3 Feeding regime for crumble to KGW larvae in rearing tanks

Days Post Hatch	Size Crumble
65	300µm
75	400µm
100	400/800µm
114	800µm
128	800µm/1mm
135	1mm

Water temperature

Refer to Table 7-2.

Grading

It is not necessary to grade KGW to reduce cannibalism, as is the case for other highly predatory species (ie. snapper, mulloway and yellowtail kingfish). The absence of cannibalism in KGW is an advantage for hatchery production as survival is high after feeding is established. However, grading larvae is advised as it can assist with management of weaning. Smaller juveniles can take a while to start feeding on different sizes of crumble and pellet, as they are dominated by larger juveniles. Separating different sizes of juveniles will result in improved growth rates and assist in feed management. Bar graders (Figure 7 - 21 and 7 - 22) suspended in tanks are a useful method of grading fish. These graders allow small fish to fall between accurately spaced bars. Larger fish are retained in the grader and transferred to another tank. It is best to partially sedate fish with clove oil (Section 4.3.3) during this procedure.

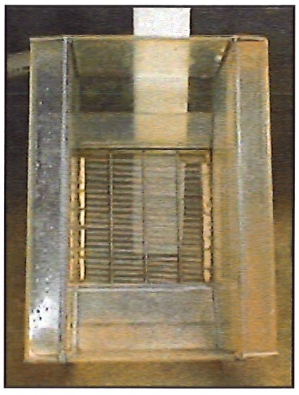


Figure 7 - 21 Fish grader

Figure 7- 22 Fish grader

Chapter 8: Nursery/growout

8.1 Operational Procedures

The FRDC project 'Spawning and larval rearing research on King George whiting *(Sillaginodes punctata)* relevant to aquaculture and fisheries biology' focused only on the larval phase of KGW production. However, the growth rate of juveniles through to market size is crucial when examining the feasibility of KGW as a commercial aquaculture species. Therefore, preliminary trials examined the growth rates and food conversion rates that could be achieved at different water temperatures throughout the growout period.

Length and weight data collected from juvenile KGW grown out at SAASC from 5 months until 14 months old is shown in Figure 8 - 1.

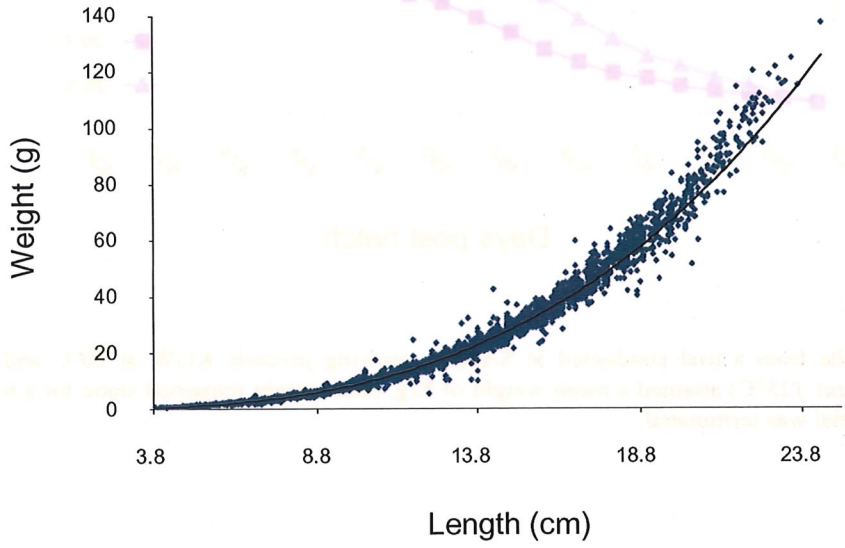


Figure 8 - 1 Length (cm) and weight (g) data collected from 4436 juvenile KGW cultured in tanks at SAASC ranging in age from 5 to 14 months old ($R^2 = 0.9912$)

Current research in South Australia is evaluating the growth rates of KGW juveniles cultured in commercial grow out systems. Juvenile's (1g) have been stocked into a saline pond at Port Augusta and in tanks using recirculating saline groundwater at Tickera and Cooke Plains. Growth data is

being collected from each site monthly and will be collated to determine realistic growth rates and the economic viability of KGW growout.

Tanks

Juveniles at SAASC were grown out in round FRP tanks (600L–10kL) in both recirculating and flow through systems. Tanks were covered with nets to prevent fish jumping out. Research has found that juveniles tend to out grow deep narrow tanks (ie. high ratio depth to surface area) experiencing specific growth rates (SGR) close to 0 % per day (Figure 8 - 2 below). This suggests that although the overall biomass of the tank is low, the bottom-dwelling nature of KGW can restrict their growth in narrow tanks. Ideally juveniles are grown out in round wide tanks (ie low ratio of depth to surface area) (5kL–10kL).

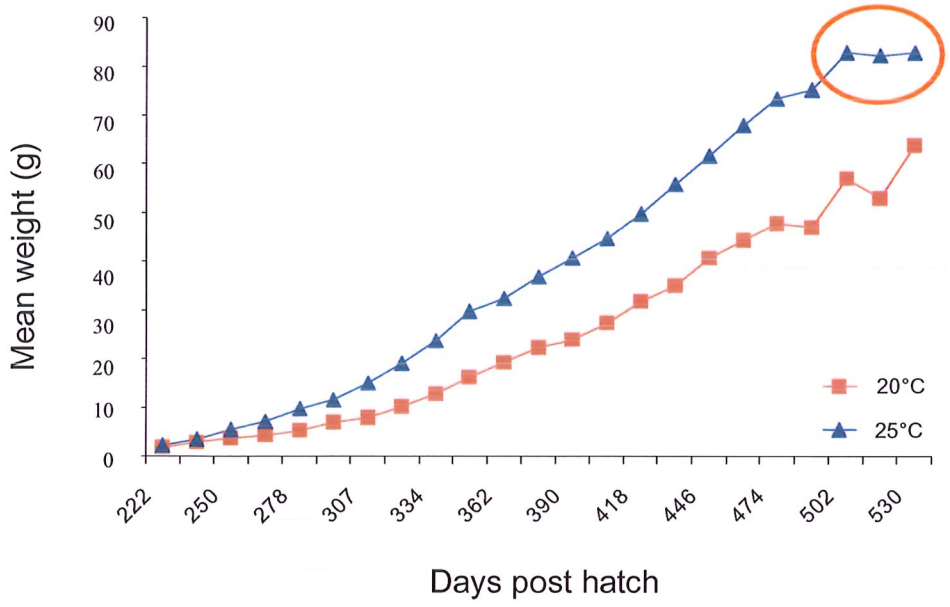


Figure 8 - 2 Results from a trial conducted at SAASC, growing juvenile KGW at 20°C and 25°C. Once juveniles in treatment (25°C) attained a mean weight of 82g, their weight remained static for a 6-week period (circled) until the trial was terminated

Aeration

Multiple airlines with fine bubble diffusers and spray bars placed across the width of the tank are used in juvenile tanks to maintain DO2 (Figure 8 - 3). Levels of DO2 should be checked regularly, particularly in tanks with elevated water temperatures and high stocking density. It is important to maintain DO2 levels at as close to 100% saturation (Section 11.8) as possible to optimise growth performance.

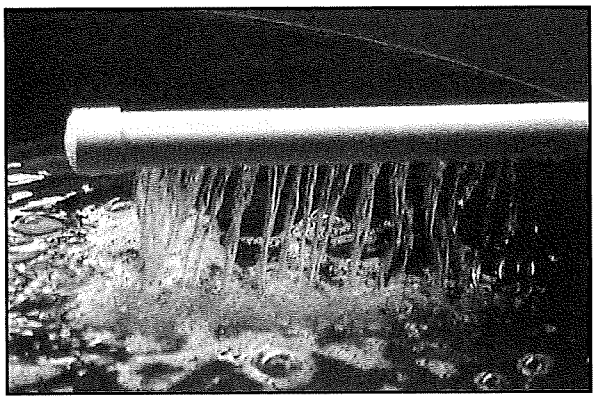


Figure 8 - 3 Spray bars used in rearing tanks

Lighting

Tanks are exposed to a combination of light and dark periods (14 hours light: 10 hours dark). Avoid strong fluorescent lights. Light level should be low, as KGW are typically "flighty" fish.

Stocking density

Juveniles' (1–50g) are stocked into tanks at a density between 2–5 kg/m3. Stocking density will primarily be determined by the ability of the culture system to maintain high water quality (i.e. DO2 level, ensure waste removal) as demands of feeding and metabolism increase.

Counting/estimating fish numbers

To effectively manage tanks, it is important to maintain accurate records numbers and average weight (g) of fish in tanks. Several methods are used to count fish: by hand or using commercial fish counters. Estimate may also be achieved by using a mean of a number of sub-samples.
Counting individual fish is stressful for the fish and impractical with large numbers. Fish counters are expensive and can still be quite time-consuming.

The most practical, cost-effective method, especially with large numbers, is to estimate the number of fish based on the mean of a number of sub-samples. This should be determined when stocking each tank. An ongoing record of mortality is then used to allow for changing numbers in each tank. Regular weight checks should be used to monitor growth performance and adjust feeding based on total fish biomass (ie. total number of fish x average weight) in each tank.

Procedure for estimating fish numbers:

- Partially sedate fish (Section 4.3) so that they are manageable.
- Record the weight (g) of fish within a sub-sample of at least 20–40 fish by placing fish in a tared container of seawater on an electronic balance.
- Repeat the above steps weighing 5 sub-samples in total
- Calculate the mean weight (g) of the sub-sample by dividing the combined weight of the fish in the sub-sample by the number of fish in the sub-sample.
- Calculate the mean (of an individual fish) across all sub-samples by adding together the mean of each sub-sample and divide by 5 (assuming there is 5 sub-samples)
- Weigh the remaining fish by netting a group of juveniles and placing them into a tared volume/s of seawater.
- Calculate the combined weight (g) of all the fish in the tank.
- Divide the total weight by the mean weight of the sub-sample of fish.

The result is the estimate of the number of fish in a tank.

Example

The total weight of 20 fish in sub-sample 1 is 65g:

Mean weight of fish from sub-sample 1 = 65/20 = 3.25g/fish

The total weight of 32 fish in sub-sample 2 is 85g

Mean weight of fish from sub-sample 2 =85/32 =2.66g/fish

The total weight of 20 fish in sub-sample 3 is 62g

Mean weight of fish from sub-sample 3 =62/20 =3.10g/fish

The total weight of 25 fish in sub-sample 4 is 70g

Mean weight of fish from sub-sample 4 =70/25 =2.80g/fish

The total weight of 26 fish in sub-sample 5 is 72g

Mean weight of fish from sub-sample 5 =72/26 =2.77g/fish

Mean of fish across all sub-samples	=(3.25+2.66+3.10+2.80+2.77)/5	
	=2.92g/fish	
Total weight of all the fish in tank	= 4,214.2 g	
Average number of fish	= 4,214.2/2.92	= 1,443.22

There are (estimated) 1,443 fish in the tank.

8.2 Feeding strategies

Feeding can be a major cost in the production of fish, so feeding strategies are tailored to maximise the growth and survival of fish at a minimum cost. The effectiveness of the feeding regime is measured by the food conversion ratio (FCR). The FCR is defined as the unit weight of dry feed required to produce a unit weight of wet fish and is used as an index of how efficiently a fish grows (Tucker 1998). FCRs can be a useful tool in determining whether fish are being over- or under-fed. The optimum FCR that can be achieved varies between species and life stage within a species as well as the type of system and is highly dependent on the amount of nutritional work conducted to specifically design a feed for a particular species. Generally, FCRs around 1 are acceptable. Optimum diets for KGW have not yet been developed. However, research indicates that KGW fed on commercial Barramundi feed (Skretting Australia) can achieve FCRs around 1 during the nursery phase.

Figure 8 - 4 outlines the relationship between growth rate feeding rate and FCR. The FCR increases when the feeding is too low or too high. If an FCR is high and there has been a lot of feed left in the tank over that period, then it is likely that over-feeding is occurring. Conversely, high FCR can also result also result from underfeeding. If this is suspected then the feeding rate should be increased for a subsequent period and the FCR re-measured. If the FCR remains high, then a secondary factor is most likely affecting growth rates. Additional factors that affect growth rates include temperature, salinity, pH, water quality and fish stress.

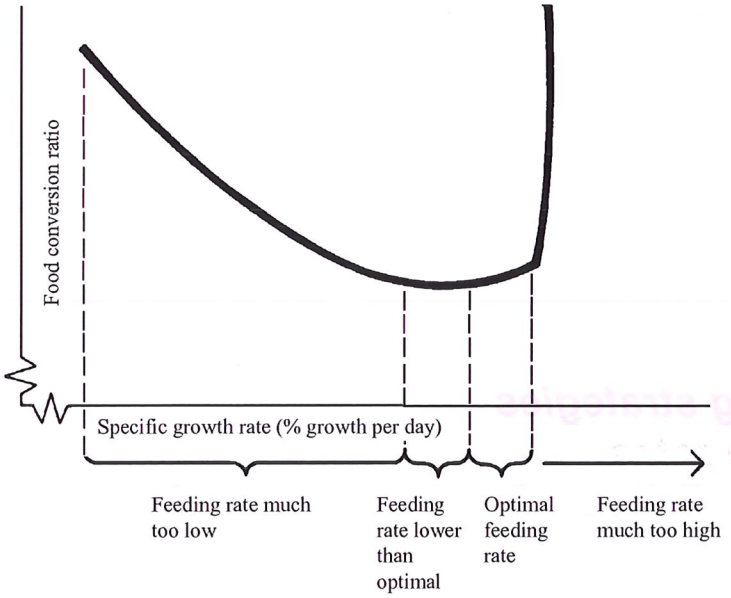


Figure 8 - 4 Relationship between growth rate, food conversion ratio and feeding rates (modified from Laird and Needham 1988)

Feed rate is generally stated as percentage biomass of fish per day (% bw/day) calculated from the number of fish and average weight records. Feeding rates change as fish develop. As the fish get larger and their growth rates decrease, and feeding rates need to be reduced. At SAASC feeding rates for KGW juveniles are based on the table below.

Table 8 - 1 Feeding rates for KGW juveniles fed on a commercial Barramundi pellet

Mean weight (g)	Size pellet	20°C (% bw/day)	23°C (% bw/day)	26°C (% bw/day)
1-2	800µm	8	8.5	9
2-3	800µm/1mm	8	8.5	9
3-6	1mm	6	7	8
6-10	1mm/1.5mm	5	5.5	6
10-16	1.5/2mm	4.5	5	5.5
16-24	2mm	4	4.5	5
24-30	2mm	4	4.5	5
30-40	2mm	3.5	4	4.5
40-60	2mm	3.5	4	4.5
60-80	3mm	3	3.5	4

Feeding intervals are spread over the duration of the light period with higher feeding frequency required for small fish. Automatic feeders that are programmed to dispense pellet several times throughout the light period can be very effective. There are many different feeders available ranging

from basic to very complex. Fish appetites will vary daily, therefore when feeding set rations fish will sometimes be overfed or underfed. To achieve optimal feeding, a combination of automatic feeding and manual feeding should be followed where automatic feeders provide 80–90% of feed (ie. base level) with the remaining feed distributed manually to accommodate daily variations in feeding response. Manually feeding tanks a couple of times a day ensures that fish will always be fed to satiation.

Always store feed in airtight refrigerated containers and utilised within a few weeks.

8.3 Growth rates

Growth rates are highly correlated with temperature. Research has indicated that temperatures of 25–26°C have high specific growth rates (up to 2.5%/day). However, water quality at these temperatures can become problematic (see FRDC (www.frdc.com.au) final report 1997/314 'Spawning and larval rearing research on King George whiting (*Sillaginodes punctata*) relevant to aquaculture and fisheries biology' for complete review of trials conducted). It may therefore be more practical to operate tanks at around 23°C to achieve higher growth rates while maintaining water quality. Poor water quality, low DO2 levels and inadequate feeding, stress fish and severely reduce growth rates.

Grading

Juveniles are graded as they enter the nursery phase. Separating the fish according to size classes allows feeding rates and size of crumble/pellet to be more accurately estimated. See 'Grading' in 'Clear water' phase (Section 7.4.2: Larval rearing).

Chapter 9: Health and Diseases

9.1 Identification

Successful management of health and diseases of fish stocks is a three-step process.

- Identification (recognise and identify a problem),
- treatment (if available), and
- prevention (identify the cause of the problem and take steps to avoid a recurrence).

The most important step in managing healthy fish stocks is to recognise a problem. Fish should be visually monitored regularly for unusual behaviour and/or symptoms. If unusual behaviour is observed, then individual affected fish should be examined. If no specific disease or health concern can be identified, then moribund or dead fish should be submitted for veterinary pathology diagnosis and transported using the method recommended by the vet.

9.2 Treatment

Once the health concern/disease has been identified, a treatment (if available) can be administered. A veterinary pathology service (VPS) or a veterinarian who specialises in aquaculture can advise on treatments for a specific disease. **Note: Do not use any chemicals mentioned in this manual without approval of the relevant state and federal authority. Please contact the National Registration Authority (NRA) on (02) 6272 5852 or access their website www.nra.gov.au for current permit status.**

9.3 Prevention

After a disease has been identified and treated (if possible), the cause of the disease also needs to be identified to prevent possible future recurrence. Additional stresses (ie. poor water quality, inadequate feeding, high stocking densities) should be avoided as these can cause disease outbreaks.

Below is a table of some common diseases/health concerns found in captive KGW stocks.

Table 9 - 1 Common symptoms and health concerns/diseases experienced in KGW stocks held at SAASC and possible treatments

Symptoms	**Possible health concern/disease**	**Treatment (contact NRA for current permit status)**
Crowding of fish at the water inlets or airlines. Fish gulping at the surface of tanks. Gills flared.	Low DO_2 levels.	Increase DO_2 levels by various aeration methods (sprayers, airstones, porous tubing or oxygen injectors).
Large amounts of foam on the surface of tanks. Excess production of mucus on the gills of fish. Gills flared. Fish dark in colour.	Irritation on the gills of fish possibly from flukes (digenean flukes), parasites or chemicals. Take a gill scrapping (*see* below) to check for flukes or parasites.	50ppm formalin and 0.25pp Neguvon as a static bath for about 4 hours.
Fish begin to 'flash' (swim to the bottom of the tank and turn over, exposing their underside). Excess production of mucus on the body of the fish.	Water quality problems or parasitic diseases infecting fish. Take a gill/skin scraping (*see* below) to check for parasites.	50ppm formalin and 0.25pp Neguvon as a static bath for about 4 hours.
White nodules up to 1mm in diameter forming under the skin of the fish. Extensive skin necrosis and subsequent secondary infections.	Parasites. *Cryptocaryon* irritations	Copper sulphate to break the lifecycle. Kills the swarmers (tomites) produced from Cryptocaryon encysts (Moller and Anders 1986).
Lethargic fish. Poor reflexes. Fish dark in colour.	Bacteria *Vibrio alginolyticus* *Pseudomonas aeruginosa*	A form of Tetracycline (oxytetracycline). A series of five, 4-hour static baths.

Symptoms	Possible health concern/disease	Treatment (contact NRA for current permit status)
Disorientation. Fish swimming in circles or away from the 'pack'. Lethargic fish. Restricted or limited movement in fish. Ulcers of any part of the fish (Figure 9 - 1). Haemorrhaging on the underside of fish.	Fungi. *Exophiala salmonis*. Note: to positively identify this disease, samples need to be sent to a VPS or veterinarian.	No current treatment. Euthanase suspect fish. Avoid additional stresses.
A marked reduction in feeding activity. Scale protrusion and rough textured skin.	Starvation, possibly as a secondary symptom to a previous infection. If no obvious disease/infection can be identified then a fish sample should be sent to a VPS or a veterinarian.	See a VPS or a veterinarian regarding disease and treatment.

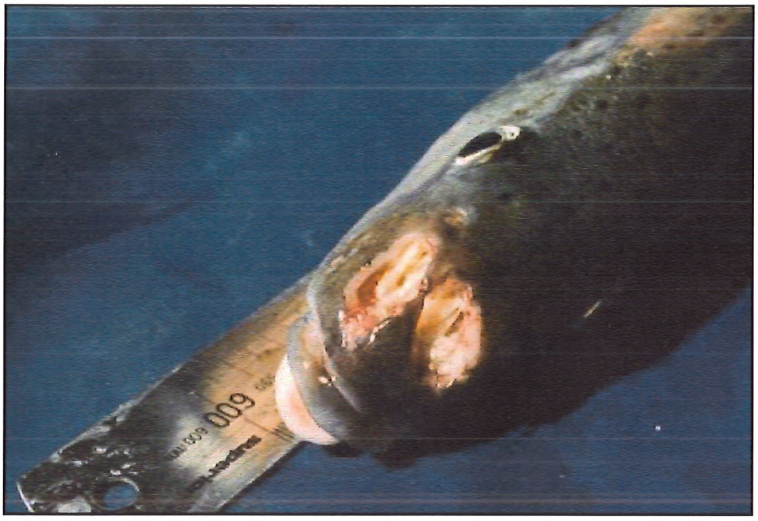


Figure 9 - 1 Adult KGW infected with *Exophiala salmonis*

Biopsy gill/ skin scrapings

Skin scrapings can be performed by gently scraping the surface with a small glass slide. Gill biopsies are taken by snipping a few complete gill filaments from the gill arch of fully anaesthetised or dead fish. Scrapings are placed on a microscope slide with a small amount of saline water. A cover slip is placed over the slide and examined under a compound microscope. These methods can be used to reveal the presence of external skin parasites (*Cryptocaryon* irritations) on gill flukes.

Chapter 10: Live Feeds

10.1 Micro-algae

Micro-algae are single-celled plants that range in size from 2 to >100µm, with more than 40 species used in aquaculture worldwide (Lavens and Sorgeloos 1996). Micro-algae serve the following functions in finfish culture:

- provide feed for cultures of rotifers, copepods and *Artemia,*
- stabilise water quality,
- provide general nutrition for larvae via live foods,
- increase visual contrast of live foods in larval rearing tanks, and
- assist with microbial control during the 'green water' phase of larval rearing.

Micro-algal species are generally selected due to:

- hardiness during mass culture (ie. wide-ranging tolerance to environmental parameters),
- nutritional value to predator,
- ability to be cultured at a low cost,
- suitable cell size, and
- digestibility.

At SAASC, the following main species are cultured for marine finfish operations: *Nannochloropsis occulata*, *Tetraselmis suecica*, *Isochrysis galbana* (T.Iso) and *Pavlova lutheri*. Generally, a hardy micro-algae (*Nannochloropsis occulata*) with a relatively low nutritional profile is used to maintain stock cultures of rotifers. Algae with higher nutritional content (*Tetraselmis suecica*, *Isochrysis galbana* (T. Iso) and *Pavlova lutheri*) are used for boosting rotifer quality prior to feeding to larvae.

Ideally, a mix of micro-algal strains should be used in tanks, to provide a balanced nutritional profile (Section 7.4).

The culture of micro-algae can be labour-intensive and can be the limiting factor in the production of live feeds. Consequently, artificial supplements or replacements (preserved micro-algae pastes, micro-encapsulated diets and yeast-based diets) can be used. This is not a regular practice at SAASC (for more information on alternatives to micro-algae see Lavens and Sorgeloos 1996) as the research scale operation is adequately supplied from available micro-algal culture facilities (total 20kL).

10.1.1 Micro-algal growth

Algae has five distinct phases of growth (Figure 10 - 1). Understanding and identifying these stages will assist with maximising algal production and longevity of cultures.

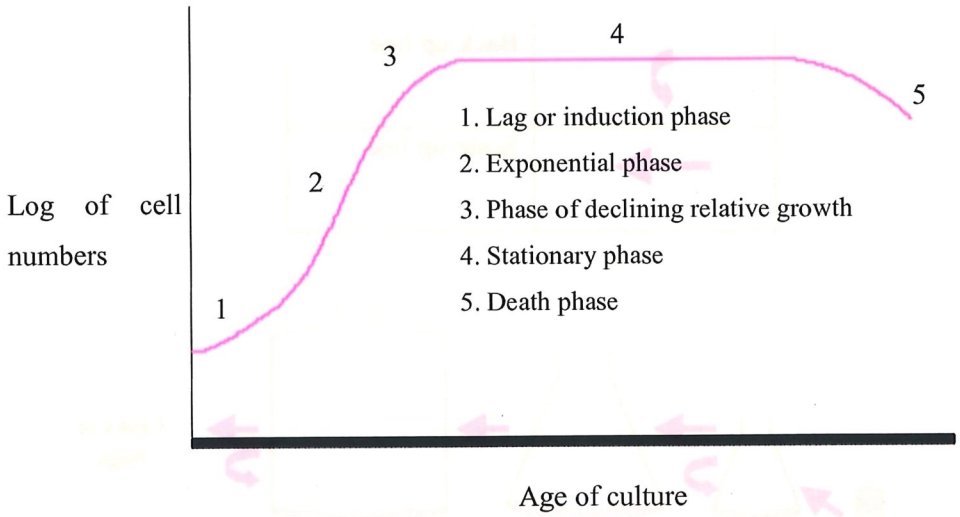


Figure 10 - 1 Five phases of algal growth (from Lavens and Sorgeloos 1996)

Lag phase: period during which there is little increase in cell density following inoculation.
Exponential phase: cell density increases as cells rapidly divide.
Phase of declining growth rate: cell division slows down when nutrients, light, pH, carbon dioxide or other chemical factors begin to limit growth.
Stationary phase: cell density remains constant, where the level of limiting factors is enough to sustain the current cell density, but not enough to support further increases in cell density.
Death or 'crash' phase: nutrients are depleted, water quality deteriorates and there is a rapid decline in algae cell density.

Algae should be maintained in the exponential phase. Cultures in the death/crash phase should be disposed of. To maintain algae in the exponential phase, it is necessary to regularly harvest algae and add nutrients and vitamins to maintain culture growth.

10.2.2 Micro-algal protocol

At SAASC, micro-algae are produced from small volume (mL) starter cultures and progressively scaled up to inoculate large volume (100-1000s L) tanks or bags. Axenic (bacteria-free) starter cultures (20 or 100mL) can be obtained from CSIRO (Hobart, Tasmania) or larger volumes (3L+) non-axenic starter cultures can be obtained from SARDI (Adelaide, South Australia). A starter culture is split between two 250mL conical flasks. One flask is maintained as a back-up, the other is systematically scaled up as shown in Figure 10-2. At SAASC, 4 back-up 250mL cultures are maintained for each species. One back-up culture is discarded and replaced at each weekly transfer.

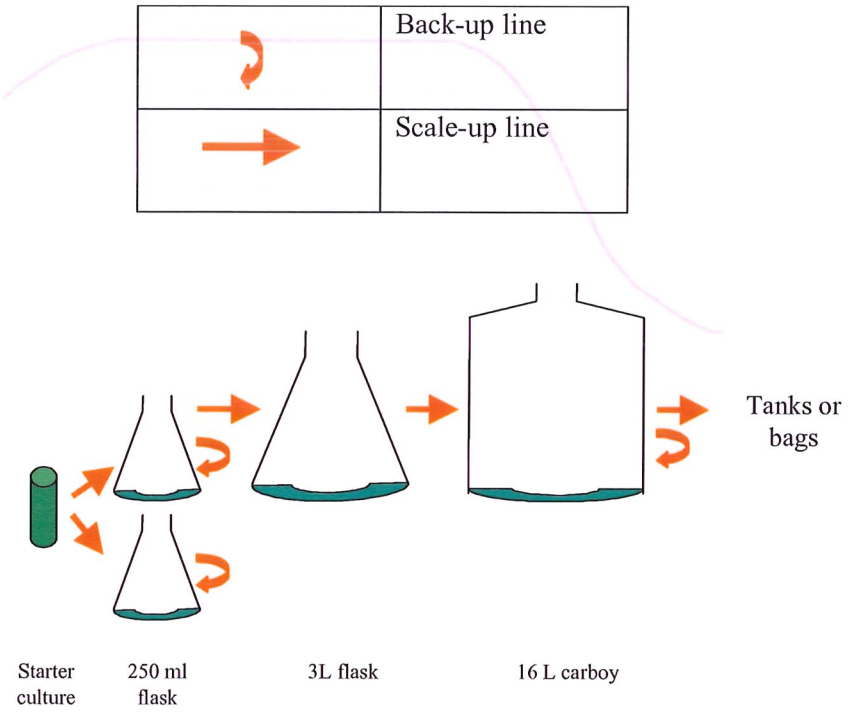


Figure 10 - 2 Scale-up production of micro-algae at SAASC

Aseptic techniques

The procedure outlined below should be followed during the inoculation of micro-algal flasks (250mL and 3L).

- Perform transfers in a clean environment, if possible in a laminar flow cabinet.
- Wipe all surfaces down with alcohol before use. Spray hands with alcohol before handling flasks.
- If using a laminar flow cabinet, always place new cultures towards the back of the cabinet.
- Flame the necks of both new and old flasks, before and after adding inoculum.
- Flame bungs before placing them in new flasks.

General hygiene and management

- Autoclave all equipment used for flasks and carboys at 120°C for 20 mins.
- Keep all pipettes in alcohol.
- Clean airlines and air stones in chlorinated water. Rinse with fresh water before use. Note: these chemicals are toxic and care should be taken when using them.
- Wash tanks with chlorinated water and then rinse in fresh water. A quick effective method of achieving this is to have a bucket containing chlorinated water in the micro-algae room. Dip the broom in the bucket and brush up all sides of the tank covering the total surface area of the tank. Leave for approximately 15–30 mins. Rinse with fresh water until there is no chlorine odour.Monitor pH levels of cultures weekly using pH indicator strips.
- Check for *Vibrio sp.* bacteria in flasks every 1–4 weeks using T.C.B.S plates. T.C.B.S plates contain a vibrio–specific agar medium that can be purchased from a medical supplier.

Stock cultures

A single starter culture (~20mL sterile tube) is used to inoculate 2–3 stock cultures (250mL flasks) (Figure 10 - 3). After 3–4 weeks using aseptic techniques, about 10% of the stock culture are transferred into another sterile 250mL flask. This process is repeated every 3–4 weeks.

Figure 10 - 3 Stock cultures of micro-algae

Inoculation procedure

- Prepare a 250mL flask with 100mL culture medium (Section 11.2.1for recipe) and autoclave.
- Add 10mL (approximately 10% of total volume) of micro-algae from starter culture using aseptic techniques (Section 10.1.2). Place the bung in the flask and cover with an Alfoil cap.
- Provide the flask with a constant light source. 200 lux is ideal for maintaining stock cultures, lights with higher intensity can cause micro-algae cells to reproduce rapidly, exhausting nutrients and ultimately crashing. At SAASC, illumination for stock cultures is provided by cool, white 36 watt fluorescent tubes.
- Swirl flasks daily to help keep micro-algal cells in suspension.
- Do not keep flasks longer than 3–4 weeks.Flasks should be maintained at 20°C.

3L flasks

A single 250mL flask (Figure 10 - 4) is used to inoculate one 3L flask and one 250mL flask.

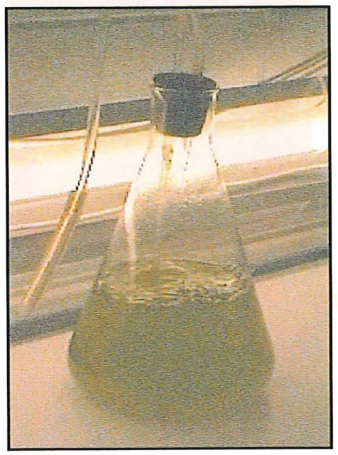


Figure 10 - 4 3L culture of micro-algae

Inoculation procedure

- Prepare a 3L flask with culture medium (Section 11.2.2 for recipe) and autoclave.
- Add about 80mL of micro-algae from a 250mL flask, using aseptic techniques (*see* section 10.1.2 for aseptic techniques). Place a bung in the flask and cover it with an Alfoil cap.
- Aerate flasks through 0.2µm inline filters and place in a well lit area (~1000 lux).
- Transfer the flask after about 1 week.
- 3L flasks should be maintained at 20°C.

16L carboys

One 3L flask can inoculate either one or two carboys (Figure 10 - 5) depending on the urgency of the carboy. If only one carboy is inoculated, it will be about 7 days before it can be used, or alternatively 10–14 days if 2 carboys were inoculated (the initial algal density in these carboys is less and they therefore take longer to reach a harvest density).

Figure 10 - 5 16L carboys of micro-algae

Inoculation procedure

- Prepare 16L carboy with culture medium (Section 11.2.3 for recipe).
- Add about 2L of micro-algae from a 3L flask and seal with a bung.
- Aerate carboys vigorously with air in a well lit area (1000 lux). A mix of air and carbon dioxide can also be used to increase production and assist in controlling pH. Ideally for algae, the pH should be maintained at 7.8. If high pH is experienced, increase the CO_2. Do the reverse for low pH levels.
- Once in the exponential phase, 3L of algae from the carboy can be used to inoculate an additional carboy (prepared using the same method described above) and the remaining algae used to inoculate a mass culture tank.

Mass culture tanks

At SAASC, 1000L conical bottom and 5000L parabolic (Figure 10 - 6) FRP tanks are used for the mass culture of algae. One carboy is used to inoculate each of these size tanks, unless algae is required quickly in which instance 2 carboys are used for one tank.

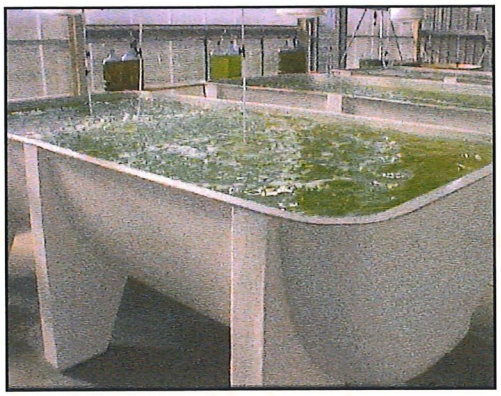


Figure 10 - 6 Mass culture tanks of micro-algae

Inoculation procedure

- Fill tanks with filtered (1µm), UV sterilised seawater.
- Chlorinate with 12.5% active sodium hyperchlorite at a rate of 0.2mL per litre, leave for 0.5-2 hours.
- Dechlorinate tank using 0.2mL 12.5% sodium thiosulphate (ie. stock solution of 125g of sodium thiosulphate in 1L distilled water) per L seawater. Aerate tank vigorously (this assists with mixing the sodium thiosulphate, removing sodium hyperchlorite and maintaining DO2 levels which can be depleted rapidly on the addition of sodium thiosulphate) for a period of 5–10 mins. Note: these chemicals are highly toxic and care should be taken when using them.Add culture medium (Section 11.2.4).
- Add 1–2 carboys of algae to each tank.Aerate tank. CO_2 is generally not used during this process although it can be useful in mass cultures with problematic pH levels (see below for more information on aeration and CO_2 levels).
- During short day length months provide additional light (1000+ lux) to achieve 12:12 or 16:8 light/dark using 400 watt metal halide lights.
- Leave for 5-7 days to reach peak of exponential phase.
- After harvesting most of the algae from the tank, refill it with 1µm filtered and UV treated seawater, nutrients and vitamins (as per inoculating procedure for tanks). Tanks can be re-started 2-3 times (4–5) before being used completely or dumped and cleaned.

Physical parameters

Temperature

Table 10 - 1 indicates the optimum and temperature range for the culture of some commonly used algal strains.

Table 10 - 1 Optimum temperature range and growth of 4 common micro-algal species (*Tetraselmis suecica; Nannochloropsis occulata; Isochrysis sp.* (T.Iso) and *Pavlova lutheri*)

Micro-algal species	Temperature range	Optimum growth in the first 7 days
Tetraselmis suecica	10°C-30°C	20°C-25°C
Nannochloropsis occulata	10°C-30°C	20°C-25°C
Isochrysis sp. (T.Iso)	15°C-30°C	25°C-30°C
Pavlova lutheri	10°C-25°C	15°C

Salinity

All the species used at SAASC (*Tetraselmis suecica, Nannochloropsis occulata, Isochrysis sp.* (T.Iso) and *Pavlova lutheri)* have a wide salinity tolerance (7 - 35‰). Cultures at SAASC are generally maintained at 35‰.

pH

For all the species mentioned above, the pH should ideally be maintained around 7.8. If pH levels start to increase (ie. approach 9) sodium bicarbonate can be added at 100mg/1000L (reduces the pH by 0.5-0.7) or alternatively CO_2 can be mixed with air.

Aeration

Vigorous aeration levels should be provided to all cultures as this will prevent cells from settling and assist with mixing cells to increasing productivity by maximising exposure of cells to light.

Light

Constant illumination is provided to stock cultures and flasks (250 mL - 3L flasks) with Philips daylight or cool, white, 36 watt fluorescent tubes. Flasks should be 0.5m from the lights to provide the flasks with approximately 200 lux.

Carboys and tanks are provided with 18 hours light using a combination of natural light and either cool white fluorescent tubes (36 watt) or metal halide lights (400 watt) to maintain 1000+ lux. For tanks larger than 1kL, two metal halide lights are provided per tank (Figure 10 - 7). Flasks and tanks are also exposed to natural light where possible.

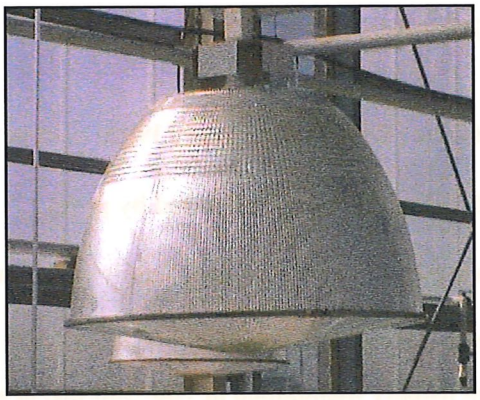


Figure 10 - 7 Metal halide lights (400 watt) used at SAASC

10.2 Rotifer culture

The recognition of rotifers *(Brachionus sp.)* as a suitable feed for marine finfish larvae occurred in Japan in the late 1960s and marked the start of successful mass larval rearing of marine finfish species (Lubzans *et al* 1989). Currently, the rotifer is regarded as the essential live prey for culture of marine finfish larvae to the extent that rotifer production capacity is a prime determinant of hatchery production (Yoshimura *et al* 1996). This is supported by personal observations during the development of commercial hatcheries in SA, where the ability to maintain high and stable production of rotifers has been critical to successful production.

Rotifers *(Brachionus sp.)* have the following characteristics, which make them an appropriate prey organism for first-feeding KGW larvae:

- small size (mean 160µm (S-type) and mean 239µm (L-type)),
- slow swimming (allowing them to be captured by prey),
- filter feeders (allowing them to be enriched, supplying larvae with specific nutrients),
- can be mass cultured in large volumes (100–100,000L) at very high densities (eg. up to 30,000/mL although usually maintained at 100–300/mL in semi-continuous culture),
- fast reproduction rate (0.7–1.4 offspring/female/day), and
- tolerance to a wide range of physical and chemical conditions (water temperature, salinity, pH and NH_2).

Many species of rotifers are found in both fresh and brackish waters. In aquaculture, a simple classification is used based on two morphotypes, namely *Brachionus rotundiformis* or small strain (S-type) rotifers and *Brachionus plicatilis* or large strain (L-type) rotifers (Lavens and Sorgeloos 1996). Both S-type and L-type rotifers are cultured at SAASC using semi-continuous culture methods.

10.2.3 Rotifer biology

The rotifer body has three distinct parts: the head, trunk and foot (Figure 10 - 8). The head carries the rotatory organ or corona, which is easily recognised by its annular ciliation, from which the name of the Rotatoria (bearing wheels) originates. The retractable beating action of fine hair-like

cilia of the corona assures locomotion and a whirling water movement which facilitates the ingestion of small particles (mainly algal cells and detritus).

The trunk contains the digestive tract, the excretory system and the genital organs. A characteristic organ for the rotifers is the mastax (a calcified apparatus in the mouth region) that is effective in grinding ingested particles, providing them with an effective mechanism to break down cell walls of algal and yeast cells before digestion.

The foot is a ring-type retractable structure without segmentation ending in one or four toes (this information is based on Lavens and Sorgeloos (1996); for more information consult this reference.).

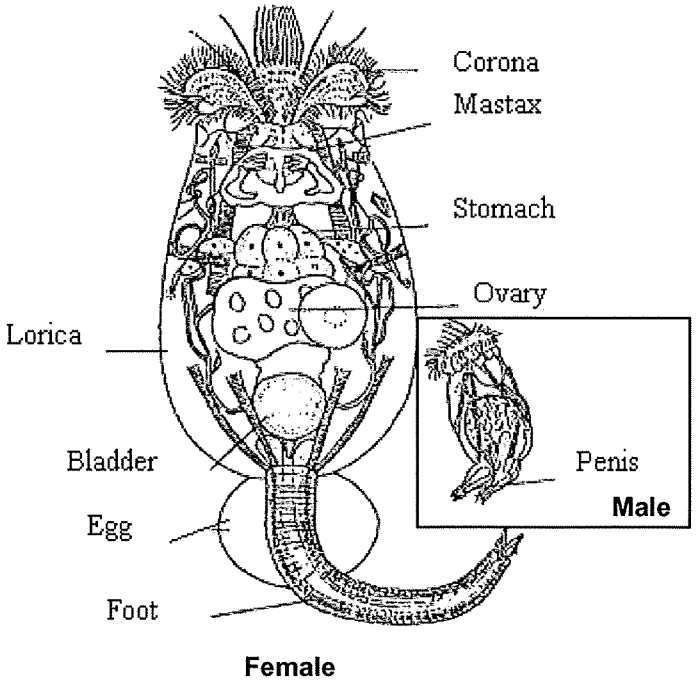


Figure 10 - 8 Internal diagram of a rotifer, *Brachionus plicatilis* (taken from Lavens and Sorgeloos 1996)

10.2.2 Rotifer reproduction

Asexual reproduction

Asexual reproduction (parthenogenesis) is the fast mode of reproduction and occurs under favourable environmental conditions (Figure 10 - 9). This involves amictic females producing amictic (diploid, 2n chromosomes) eggs, which develop and hatch into amictic females. Hatchery production of rotifers aims to maintain rotifers in this mode of reproduction to ensure ongoing mass cultures.

Sexual reproduction

When environmental conditions are less favourable (poor water quality, low temperature etc), females can switch to sexual reproduction. In this instance, females produced can be both mictic and amictic. Mictic females produce haploid eggs (n chromosome) that can either hatch out as haploid males (about a quarter the size of the female), or become fertilised resting eggs that will develop and hatch into amictic females only after exposure to specific environmental conditions. This mode of reproduction is not favourable for hatchery operations unless resting egg production is the objective.

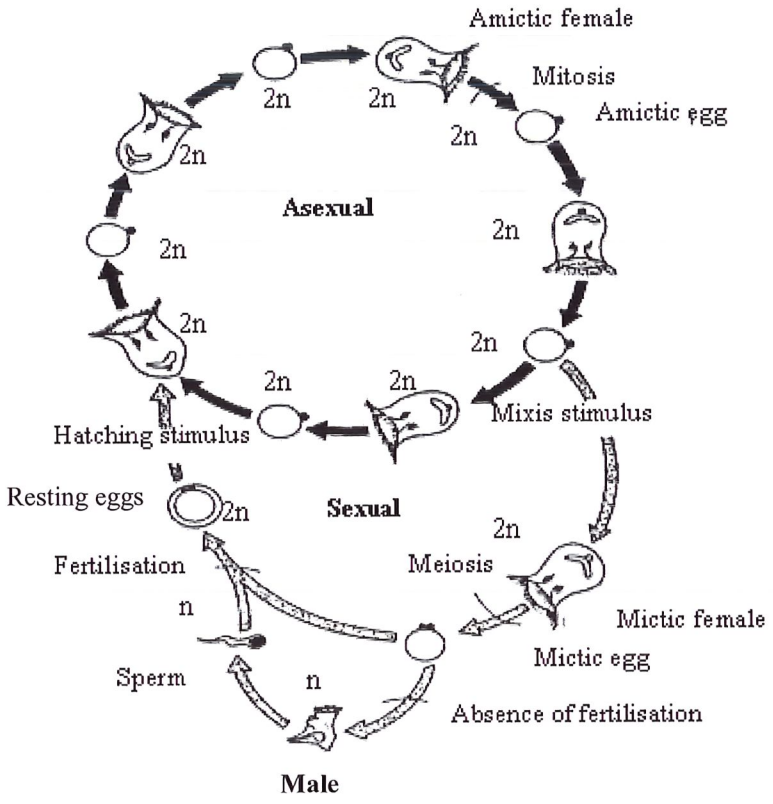


Figure 10 - 9 Rotifer life cycle (Lavens and Sorgeloos 1996)

10.2.3 Rotifer culturing protocol

SARDI uses of scale-up procedures for rotifers as practiced in most large commercial hatchery facilities (Figure 10 - 10).

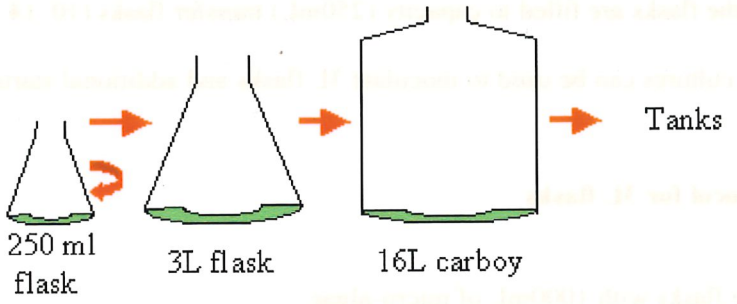


Figure 10 - 10 Scale-up production of rotifers at SAASC

Starter cultures

Starter cultures of rotifer strains are maintained in 250mL flasks, with the following advantages:

- back-up for culture washes,
- ability to maintain pure cultures of large and small strains to start clean cultures in the event of cross-contamination, and
- inoculation for new cultures during preparation for a hatchery production run.

Initial starter cultures can be obtained from the wild, from research institutes or from commercial hatcheries. Lavens and Sorgeloos (1996) outline thorough methods for disinfecting cultures. However, healthy starter cultures transferred using the following techniques have been found to be adequate at SAASC and apply to both small and large strain rotifers.

Inoculation procedure

- Prepare 250mL flasks with 100mL of aseptic micro-algae (flask algae).
- Inoculate 250mL flasks with healthy rotifers (from a starter culture) at a density as low as 2/mL. To determine healthy rotifers, place a small amount of well rinsed rotifers (to reduce ciliate and bacteria contamination) from a starter culture on a Petri dish under a dissecting microscope. Using a pipette, pick out individual rotifers that are active and

carrying eggs. This can be a difficult process which is assisted if there is only a small number of rotifers diluted in seawater. Place bungs on flasks.

- Place flasks in a well lit area (200 lux).
- Add a small amount of algae (10–15mL) and swirl flasks daily or as required.
- When the flasks are filled to capacity (250mL) transfer flasks (10–14 days).
- Starter cultures can be used to inoculate 3L flasks and additional starter cultures.

Inoculation protocol for 3L flasks

- Prepare flasks with 1000mL of micro-algae.
- Inoculate with healthy rotifers at a density of 50/ml.
- Aerate flasks through 0.2µm filters in a well lit area.
- Add 150mL of algae daily or as required.
- Once the flask is filled to capacity, transfer to a 16L carboy.

Inoculation protocol for 16L carboys

- Prepare carboys with 10L of algae.
- Inoculate with healthy rotifers at a density of 50/ml.
- Aerate carboys through 0.2µm filters in a well lit area.
- Add 1L of micro-algae daily or as required.
- Once carboys are filled to capacity, rinse rotifers thoroughly and transfer to tanks for mass culture.

10.2.4 Mass culture of rotifers

At SAASC, a range of tanks (800L–4kL) are used for mass rotifer reproduction. Generally, flat bottom tanks are suitable for batch cultures although conical bottom tanks assist removal of waste if this is done daily.

Large strain rotifer protocol

Culture tanks are half filled with 50% miro-algae and 50% 1µm filtered and UV treated seawater, typically *Nannochloropsis occulata* is used for rotifer cultures although other species such as T.Iso are also used (micro-algal cultures with densities of about 5 - 6 million cells/mL are ideal).

Multiple airlines are placed in the tank away from the walls to about two-thirds the depth of the tank. Strong aeration with large air bubbles are provided (ensure this does not physically damage rotifers) as small, fine bubbles can cause a rafting effect, removing the rotifers from suspension.
Mass culture tanks can be run at salinities up to 35ppt although salinities between 20–25ppt are more suitable for rotifers as they are brackish organisms. If the salinity in the rotifer culture is greater than 5ppt different to larval rearing tanks, rotifers are acclimatised before being added to tanks. Water temperature is maintained at 22-25°C using 3 kilowatt immersion heaters with thermostat controls. Tanks are inoculated with rotifers at a minimum density of 50/ml although 200/ml are preferred. Rotifers are fed on compressed baker's yeast *(Saccharomyces cerevisiae)* once all the algae in the tank have been consumed (see below: Feeding rotifers section). Cultures are maintained for 4–5 days until rotifer densities reach full capacity. If the water quality deteriorates during this time a water exchange is conducted. This can be done using screens (Figure 10-11 and 10-12) in the culture tank to remove water but retain the rotifers.

Figure 10 - 11 Banjo screen

Figure 10 - 12 Rotifer concentrator

The tank can then be re-filled with fresh micro-algae. Once rotifer densities are between 250–300 rotifers/mL, rotifers can be harvested. At SAASC, up to half the tank volume is harvested daily and then refilled with micro-algae. Tanks are maintained for about 5–10 days. Then the whole tank is harvested and a small volume of rotifer media is used to inoculate another tank.

Tanks are vacuumed regularly to remove debris on the bottom of the tank (aeration is turned off 5 minutes before vacuuming, allowing debris to settle).

Floc collectors (washable Dacron matting) are placed in tanks when necessary to remove waste particles in suspension. These are cleaned daily.

Small strain rotifer protocol

Small strain rotifers are cultured using the same method outlined above, but the temperature is increased to 30°C. At this temperature they have a higher metabolic rate and require greater amounts of feed. Under these conditions, water quality can quickly deteriorate and a regular water exchange may be required.

Physical parameters

Table 10 - 2 provides an indication of the optimum physical parameters for the culture of S-type and L-type rotifers.

Table 10 - 2 Optimum physical parameters for the culture of *Brachionus rotundiformis* (S-type rotifers) and *Brachionus plicatilis* (L-type rotifers)

Parameter	S-Type	L-type
Water temperature (°C)	28-32	22-25
Salinity (ppt)	25-35	25-35
pH	7.5-8.5	7.5-8.5
Ammonia (mg/L)	<1	<1
Dissolved oxygen (ppm)	>4	>4

Feeding rotifers

The preferred feed for rotifers is micro-algae as it results in a high nutritional value (for finfish larvae), doesn't pollute the water and acts a buffer to water quality changes. Due to the cost and time involved in the production of algae, its use is generally limited to starting up a new rotifer culture and enriching rotifers before feeding to fish larvae. At other times, compressed baker's yeast is the main food source for rotifers. Typically, yeast is fed at 0.8-1.5g/million rotifers/day. For improved production of rotifers the total daily feed should be split into as many small feeds as practical as yeast is not active for longer than 30 minutes after addition to the culture. The desired amount is weighed out based on daily rotifer counts, then blended in fresh water before feeding.

Formulated diets such as Culture Selco and Culture selco 3000 can also be used as a supplementary

feed or as total replacement for algae and or yeast. The diets are designed to be complete replacements for micro-algae while incorporating high levels of essential fatty acids and vitamins. Products must be used according to the manufacturer's instructions as overfeeding or incorrect use can cause rapid deterioration of the water quality. The full benefit of the products will be available to the rotifers only if it is blended for the time and speed (rpm) specified by the manufacturer. Careful attention needs to be paid to tank hygiene, water disinfection and tank water dynamics when using these products.

Enrichment of rotifers

The maximum nutritional value of rotifers can be achieved by enriching rotifers 12–24 hours before feeding to larvae. Rotifers can be enriched with high quality micro-algae or commercial enrichment diets such as Protein selco (dry product), DHA Protein selco (dry product), Selco (liquid), DHA Selco (liquid) and Super Selco (liquid). Before enriching, rotifers are harvested (using the method described below) from culture tanks and rinsed for approximately 15 minutes before transfer to enrichment tanks. Strong aeration (or oxygen if required) is maintained during enrichment to ensure that DO2 levels remain above 4ppm. It is essential that rotifers are thoroughly rinsed again after enrichment, before feeding to larvae.

Rinsing/Harvesting rotifers

There are many different types of rotifer harvesters. The harvester design can affect the percentage of rotifers lost during harvest and the time taken to harvest a tank. Several types have been trialed at SAASC and the preferred design is shown in Figure 10-13. The harvester consists of 63µm mesh screen surrounding a central cylindrical PVC frame mounted in a cylindrical tub (70L). The base of the screen is surrounded by a ring of airline to prevent rotifers building up on the screen.
A standpipe 100mm is fitted to the bottom outlet to set the operating water level.

The desired volume of a rotifer culture is harvested into the region outside the screen and water is removed through a central standpipe. Condensed rotifers (outside the screen) are rinsed by attaching a flow of fresh seawater into the region for about 15 minutes. To reduce ciliate contamination in rotifer cultures, a fresh water bath can be used but this should be for no longer than 1 minute. Rinsed rotifers can be condensed into a known volume of water (ie. 20L) by removing the standpipe. Condensed rotifers are then fed out to fish larvae.

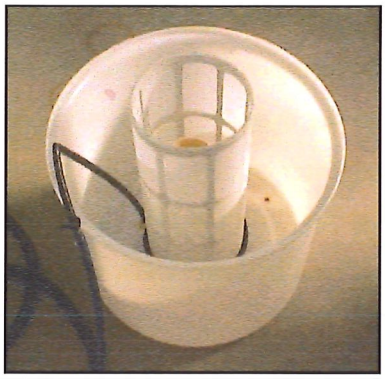


Figure 10 - 13 Rotifer harvester

Rotifer counts

Rotifer counts are undertaken daily for each culture tank to calculate feeding rates, estimate the health of cultures and determine the volumes required to harvest to provide numbers needed to feed larvae. A healthy culture has about 20% of the population carrying eggs. A volumetric method of counting rotifers is used. Ideally, the mean (average) of a minimum of 3-5 counts should be taken.

Procedure

- For each sub-sample take a 1mL sample from the tank and disperse small drops, forming a spiral or series of small rows on a Petri dish.
- Count the number of rotifers with eggs and the total number of rotifers in each drop.
- Calculate a mean for each sub-sample.
- Calculate the total number of rotifers in the tank and the percentage of rotifers carrying eggs in the tank (refer to calculations below).

Calculations

Total number of rotifers in tank = Mean no. of rotifers in 1mL sample x 1000 x volume of tank (L)

Percentage of rotifers with eggs = Mean no. of rotifers with eggs/mean total number of rotifers x 100

10.3 Artemia

The small branchiopod crustacean *Artemia* occurs in hypersaline lakes around the world, producing dormant embryos or 'cysts'. Cysts can hatch within 24 hours, producing nutritious free-swimming nauplii (Figure 10 -14) which can be fed directly to finfish larvae as a second larger live food source following from rotifers. *Artemia* use in marine finfish hatcheries is mostly:

- production of 1st or 2nd instar nauplii as a second stage live feed.

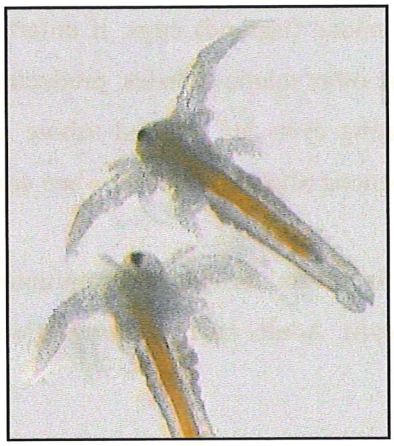


Figure 10 - 14 Enriched *Artemia* 2 dph

10.3.1 Biology and life cycle

At salinities above 150ppt Adult *Artemia* produce cysts that remain inactive for extended periods if dry. Once the cysts are immersed in seawater, they hydrate and after about 20 hours at 28°C, the cyst shell/chorion bursts, leaving the embryo surrounded by the hatching membrane. After about 4 hours the embryo breaks through the hatching membrane as a free-swimming nauplii. *Artemia* then go through a series of molt stages or instars until they become adults at about 1cm long. The first two instars are most important for production of marine finfish larvae.

First nauplii stage: (Instar I; mean 520µm long). At this stage nauplii do not ingest exogenous feed (they are feeding off the internal yolk sac) and therefore can't be enriched. First nauplii stage *Artemia* are often used to culture marine finfish larvae as they contain all the nutritional reserves at hatching and are at the minimum size. Instar I lasts for approximately 12 hours at 25°C.

Second nauplii stage: (Instar II; mean 700µm). Nauplii can ingest feeds from 1–50µm in size (i.e. algae cells, bacteria and detritus). At SAASC, only nutritionally enriched second nauplii stage *Artemia* are fed to finfish larvae as these are more easily digested and their nutritional status can be enhanced. Adults have 2 modes of reproduction:

- asexual: under favorable conditions the female produces amictic (diploid) eggs which can hatch into free-swimming nauplii which are a clone of the parent. This is the faster mode.
- sexual: females switch to this mode of reproduction under unfavorable conditions (i.e. high salinity and low oxygen levels). In this mode of reproduction (haploid) females are produced which carry mictic (haploid) eggs. If unfertilised these will hatch into males, which will then fertilise other mictic females, producing resting-stage cysts covered by a resistant chorion. Floating cysts are washed ashore under and natural conditions and eventually hatch into amictic (diploid) females when conditions become favorable.

Free swimming *Artemia* can live for several months under optimum conditions. It takes about 8 days to reach the adult stage from nauplii. Adults have a reproduction rate of up to 75 nauplii or cysts every each day.

10.3.2 Culturing *Artemia*

De-capsulating cysts

De-capsulating cysts (removing the hard brown layer the chorion) is performed to ensure maximum hatch rates within a known time frame (24–36 hours) and to reduce the bacterial load entering the larval rearing tank via *Artemia* nauplii. This process involves oxidising the resistant chorion using a strong chlorine solution (sodium hypochlorite). It is possible to hatch *Artemia* nauplii without de-capsulating. However, the hatch rate can be variable producing a size range of *Artemia* nauplii at harvesting. It can also be difficult to completely separate unhatched cysts and nauplii without de-capsulation. Cyst fragments which may be mixed with nauplii and enter larval rearing tanks can harbour bacteria and be harmful to larvae if ingested (Jenkins *et al* 1999).

Procedure for de-capsulating 100 grams of cysts

- Weigh out 100g of cysts.

- Place cysts in a beaker and fill with fresh water (fresh water due to osmotic pressure will hydrate cysts quicker), stir to ensure that all of the cysts are exposed to the water and maintain slow aeration if possible.
- Leave to hydrate for 1 hour.
- Dissolve 15g of sodium hydroxide pellets in 980mL of fresh water and chill solution on ice.
- Once all the cysts are hydrated, drain cysts using a 100µm screen and place in a large container (5–10L) with the chilled sodium hydroxide solution.
- Add 420mL of sodium hyperchlorite (12.5% active).
- Stir vigorously with a glass or plastic rod. As the reaction starts, heat will be given off, a froth will form and cysts will turn white/grey and then finally orange, indicating that the chorion has been removed. Do not allow the temperature of the solution to exceed 37°C during de-capsulation. Chilling the sodium hydroxide solution will assist in reducing the temperature. If the temperature still exceeds 37°C, then the de-capsulation solution will have to be submerged in a chilled tub during de-capsulation. Alternatively, ice or ice packs can be added.
- Once the cysts have turned orange they should be rinsed through a 100µm mesh screen under a constant flow of water to stop the reaction (wash thoroughly until no froth remains).
- To ensure all chlorine is removed, cysts are washed in 0.1% sodium thiosulphate solution for 1 minute and then rinsed again in fresh water.
- Transfer cysts into *Artemia* hatching tanks or store in the refrigerator in a small amount of seawater for up to 1 week.
- Use caution, wear gloves when handling sodium hydroxide and sodium hyperchlorite and Ensure that the de-capsulation of *Artemia* cysts occurs in a well-ventilated area.

Hatching tanks

At SAASC, cysts are incubated in 330L conical-bottom white FRP tanks (Figure 10 - 15). These tanks allow cysts and dead nauplii to be easily separated from nauplii.

Hatching tanks are pre-filled with seawater (20–35ppt). Tank temperature is maintained between 25–28°C. Strong aeration is provided in the centre and at the sides of the tank, keeping cysts in

suspension and maintaining DO2 levels above 4mg/L. During incubation, pH levels are kept between 7–8.5 with an optimum pH of 8.0. If the pH levels drop, 2.0g/L of sodium bicarbonate can be added. Light is required throughout the hatching process and is provided by an incandescent light above each tank. For de-capsulated cysts, 50–100 lux at the water surface is optimal for hatching. Cysts are stocked into hatching tanks at a density of 1–2g cysts/L (do not exceed 3g cysts/L).

Figure 10 - 15 *Artemia* hatching tank

Rinsing cysts

It is essential to rinse *Artemia* after hatching to remove glycerol and other metabolites released from cysts during hatching (Lavens and Sorgeloos 1996), which can act as a food source for bacteria. Poor rinsing of cysts can lead to very high bacterial populations during enrichment, which can be transferred to larvae, causing mortalities. Once nauplii are harvested, they are thoroughly rinsed by attaching a flow of filtered (5–30µm) seawater into the outside of the *Artemia* harvester for 20-30 minutes.

Boosting/enriching Artemia

The nutritional status of *Artemia* nauplii is highest immediately after hatching but decreases rapidly as *Artemia* invest their energy reserves in growth. Even at hatching, the level of essential fatty acids (EFA) can vary from strain to strain and even from batch to batch, affecting the growth rate and survival of larvae. Therefore, *Artemia* are enriched to achieve optimum nutritional quality. Enrichment can occur from Instar II (about 12 hours post hatch) onwards. From this stage, *Artemia* can ingest particles from 1–50µm in size. Nutrition enrichment can be achieved by:

- feeding nauplii on micro-algae with a high nutritional value (e.g. T.Iso, *P.lutheri*), or
- using commercial enrichment diets i.e. DC DHA Selco®, Super Selco®, Algamac 2000®, Progression®, Super *Artemia*®, Rich Range®, Salt Creek Range®.

To enrich *Artemia* with algae, add nauplii II with aerated enrichment tanks pre-filled with micro-algae (T.Iso or *Pavlova lutheri*). Place *Artemia* in aerated enrichment tanks for 12–18 hours. For commercial products, place *Artemia* in an aerated enrichment tank and prepare according to the manufacturer's instructions. Leave for 12–18 hours.

Harvesting Artemia

A number of different types of *Artemia* harvesters are available. The amount of *Artemia* to be harvested can affect the type selected. A suitable harvester for a range of *Artemia* volumes is the same design as the rotifer harvester (Section 10.2.4) but the mesh size of the screen is increased to 150µm.

Harvesting procedure:

- Remove air from tanks and leave for 5 minutes, allowing dead nauplii or unhatched cysts to sink to the bottom.
- Open the bottom valve and remove any dead nauplii or unhatched cysts (do not attach the harvester while doing this).
- Attach the harvester and continue to drain the tank.
- Once *Artemia* have been harvested, thoroughly rinse before feeding to fish larvae.
- Condense *Artemia* in the bottom section of the harvester and feed out to fish larvae.

Separation of cysts (where de-capsulation of cysts has not occurred)

To separate cysts from nauplii where tanks have been stocked with non-decapsulated cysts, switch off air, cover the top of the tank and leave to stand for 5 minutes. If the tank is lighter at the bottom than the top (as with a white-bottom tank) the nauplii will swim to the bottom and the cysts will float. The nauplii can then be harvested from the bottom tap (as described above) until only cyst fragments remain in the tank at which point the tap is closed. Cyst fragments can then be drained into a separate container for disposal.

Feeding Artemia to larvae

It is best to feed out *Artemia* to larvae several times a day. The easiest way is to harvest the whole day's supply of *Artemia*, keep it in a known volume in the refrigerator and feed out the portion required over several feeds (2–3 a day). As larvae grow, adjustments to the amount of *Artemia* cysts required can be assessed based on the rate of consumption of nauplii in larval-rearing tanks. The optimal amount to feed should be an amount that remains visible in the tank of fish larvae for about 30 minutes for KGW (this time will be much shorter for more aggressive feeding species).

Counting Artemia

It is advisable to determine the number of *Artemia* nauplii produced per gram of cysts hatched as this will vary depending on grade and brand of cysts and hatching conditions at each location. Follow the procedures to hatch cysts then count the number of nauplii in at least 5 x 1mL samples. The mean of these counts can then be used in combination with the tank volume (L) and the amount of cysts per gram hatched to determine the yield of nauplii per gram, as follows:

Mean number of nauplii per mL x 1000 x tank volume (L) = total number of nauplii yield

Number of nauplii hatched per gram of cysts = total number nauplii/grams of cysts stocked

This figure can be used to calculate the amount of *Artemia* required to achieved desired feeding rates (eg. 0.5–1.0 nauplii/mL) in larval rearing tanks and to project feeding requirements (ie. gram of cysts to hatch) in combination with feeding observations. The known amount of hatched *Artemia* nauplii is generally suspended in a known volume of seawater and distributed volumetrically into each larval rearing tank.

Chapter 11: Appendix

11.1 Profile of algal strains

Table 11 - 1 Comparisons of different micro-algal strains commonly used in aquaculture (modified from Lavens and Sorgeloos 1996)

Concentrations of chlorophyl a, protein, carbohydrate and lipid in commonly used species of microalgae in aquaculture					
Algal taxa	Dry weight (picogram.cell^{-1})	Chl a	Protein	Carbohydrate	Lipid
		Weight of constituent (picogram. Cell^{-1})			
Eustigmatophyceae					
Nannochloropsis occulata	6.1	0.054	2.1	0.48	1.1
Prasinophyceae					
Tetraselmis chui	269.0	3.83	83.4	32.5	45.7
Tetraselmis suecica	168.2	1.63	52.1	20.2	16.8
Prymnesiophyceae					
Isochrysis galbana	30.5	0.30	8.8	3.9	7.0
Isochrysis aff. galbana (T. Iso)	29.7	0.29	6.8	1.8	5.9
Pavlova lutheri	102.3	0.86	29.7	9.1	12.3
Pavlova salina	93.1	0.34	24.2	6.9	11.2
	Percentage of dry weight				
Eustigmatophyceae					
Nannochloropsis occulata	6.1	0.89	35	7.8	18
Prasinophyceae					
Tetraselmis chui	269.0	1.42	31	12.1	17
Tetraselmis suecica	168.2	0.97	31	12.0	10
Prymnesiophyceae					
Isochrysis galbana	30.5	0.98	29	12.9	23
Isochrysis aff. galbana (T. Iso)	29.7	0.98	23	6.0	20
Pavlova lutheri	102.3	0.84	29	9.0	12
Pavlova salina	93.1	0.98	26	7.4	12

11.2 Algal culture mediums

11.2.1 250 mL flask culture medium

Mix in 3L container and pour 100mL into each 250mL flask.

2.5 L	1µm filtered, UV sterilised seawater
0.5L	Distilled water
0.3mL	Vitamins (Section 11.5)
3mL	Erbschreiber's (Section 11.3)

11.2.2 3L flask culture medium

2.1L	1µm filtered, UV sterilised seawater
0.4L	Distilled water
3mL	Erbschreiber's (Section 11.3)
0.3mL	Vitamins (Section 11.5)

11.2.3 16L carboy culture medium

15L	1µm filtered, UV sterilised seawater
15mL	Erbschreiber's (Section 11.3)
1.5mL	Vitamins (Section 11.5)

11.2.4 Mass culture medium (using Erbschreibers)

0.5mL / L	Erbschreiber's (Section 11.3)
0.01 mL / L	Vitamins (Section 11.5)
0.005 mL /L	Trace metals (Section 11.4)

11.2.5 Mass culture medium (using Aquasol)

0.05g /L	Aquasol
0.01 mL/L	Vitamins (Section 11.5)
0.005mL/L	Ferric Chloride (Section 11.6)
0.005mL/L	Trace metals (Section 11.4)

11.3 Nutrient Erbschreiber's (minus soil extract)

Ferric chloride ($FeCl_3.6H_2O$)	1.3g
Manganous chloride ($MnCl_2.4H_2O$)	0.4g
Boric acid (H_3BO_3)	33.6g
EDTA disodium salt $2H_2O$	45.0g
Sodium di hydrogen orthophosphate ($NaH_2PO_4.2H_2O$)	20.0g
Or (NaH_2PO_4)	
Sodium nitrate ($NaNO_3$)	100.0g
Trace metals (Section 11.4)	1ml (after making it up to 1 litre)

Make up to 1 litre with boiled rain or distilled water. Autoclaving is not essential although it can help to dissolve some of it.

Add at 1 mL/L of culture for carboys or 0.5ml/L for open tank cultures.

11.4 Trace metals

Zinc chloride ($ZnCl_2$)	2.1g
Cobaltous chloride ($COCl_2.6H_2O$)	2.0g
Ammonia molybdate ($(NH_4)6MO_7O_2.4H_2O$	0.9g
Copric sulphate ($COSO_4.5H_2O$)	2.0g
Concentrated HCl	10ml
(only need a few drops until solution goes clear)	

Make up to 100ml with fresh water (this may need to be dissolved).

Add 0.005 mL/L of algal culture.

11.5 Vitamins

Allows for a loss of up to 90% vitamins during autoclaving.

B12	50mg
B1	1.0g

Dissolved in 1L of distilled water.

Add 0.1 mL/L of flask culture (before autoclaving).

11.6 Ferric chloride

Dissolve 1.3g ferric chloride in 100ml distilled water and mix.

Add 0.005 mL/L of algal culture.

11.7 Sodium thiosulphate

Dissolve 125g of sodium thiosulphate in 1L of distilled water. Mix over a heat pad using a magnetic stirrer until dissolved.

Add at a 1:1 ratio with sodium hyperchloride for dechlorination.

11.8 Dissolved oxygen

Table 11 - 2 Air solubility of oxygen (mg/L) in seawater (0-40g/kg,‰) (based on Benson and Krause 1984 as cited in Creswell 1993)

Salinity (g/kg)									
Temp (°C)	0	5	10	15	20	25	30	35	40
0	14.621	14.120	13.636	13.167	12.714	12.277	11.854	11.445	11.051
1	14.216	13.733	13.266	12.815	12.378	11.956	11.548	11.154	10.773
2	13.829	13.364	12.914	12.478	12.057	11.650	11.256	10.875	10.507
3	13.460	13.011	12.577	12.156	11.750	11.356	10.976	10.608	10.252
4	13.107	12.674	12.255	11.849	11.456	11.076	10.708	10.352	10.008
5	12.770	12.352	11.947	11.554	11.175	10.807	10.451	10.107	9.774
6	12.447	12.043	11.652	11.272	10.905	10.550	10.206	9.872	9.550
7	12.139	11.748	11.369	11.002	10.647	10.303	9.970	9.647	9.335
8	11.843	11.465	11.098	10.743	10.399	10.066	9.744	9.431	9.128
9	11.559	11.194	10.839	10.495	10.162	9.839	9.526	9.223	8.930
10	11.288	10.933	10.590	10.257	9.934	9.621	9.318	9.024	8.739
11	11.027	10.684	10.351	10.028	9.715	9.412	9.117	8.832	8.556
12	10.777	10.444	10.121	9.808	9.505	9.210	8.925	8.648	8.379
13	10.537	10.214	9.901	9.597	9.302	9.017	8.739	8.470	8.210
14	10.306	9.993	9.689	9.394	9.108	8.830	8.561	8.300	8.046
15	10.084	9.780	9.485	9.198	8.921	8.651	8.389	8.135	7.888
16	9.870	9.575	9.289	9.010	8.740	8.478	8.223	7.976	7.737
17	9.665	9.378	9.099	8.829	8.566	8.311	8.064	7.823	7.590
18	9.467	9.188	8.917	8.654	8.399	8.151	7.910	7.676	7.449
19	9.276	9.005	8.742	8.486	8.237	7.995	7.761	7.533	7.312
20	9.092	8.828	8.572	8.323	8.081	7.846	7.617	7.395	7.180
21	8.914	8.658	8.408	8.166	7.930	7.701	7.479	7.262	7.052
22	8.743	8.493	8.250	8.014	7.785	7.561	7.344	7.134	6.929
23	8.578	8.334	8.098	7.867	7.644	7.426	7.214	7.009	6.809
24	8.418	8.181	7.950	7.725	7.507	7.295	7.089	6.888	6.693
25	8.263	8.032	7.807	7.588	7.375	7.168	6.967	6.771	6.581
26	8.113	7.888	7.668	7.455	7.247	7.045	6.849	6.658	6.472
27	7.968	7.748	7.534	7.326	7.123	6.926	6.734	6.548	6.366

28	7.827	7.613	7.404	7.201	7.003	6.810	6.623	6.441	6.263
29	7.691	7.482	7.278	7.079	6.886	6.698	6.515	6.337	6.164
30	7.558	7.354	7.155	6.961	6.772	6.589	6.410	6.236	6.066
31	7.430	7.230	7.036	6.846	6.662	6.483	6.308	6.137	5.972
32	7.305	7.110	6.920	6.735	6.555	6.379	6.208	6.042	5.880
33	7.183	6.993	6.807	6.626	6.450	6.278	6.111	5.948	5.790
34	7.065	6.879	6.697	6.520	6.348	6.180	6.017	5.857	5.702
35	6.949	6.767	6.590	6.417	6.248	6.084	5.924	5.768	5.617
36	6.837	6.659	6.485	6.316	6.151	5.991	5.834	5.681	5.533
37	6.727	6.553	6.383	6.218	6.056	5.899	5.746	5.597	5.451
38	6.619	6.449	6.283	6.121	5.963	5.810	5.660	5.513	5.371
39	6.514	6.348	6.186	6.027	5.873	5.722	5.575	5.432	5.292
40	6.412	6.249	6.090	5.935	5.783	5.636	5.492	5.352	5.215

11.9 Dose table; LHRHa, intraperitoneal injection

Table 11 - 3 Dose rates for intraperitoneal injection at dose rate of 10µg/kg body weight (dose rates based on solution of 1mg of LHRHa dissolved in 20mL saline (0.9%))

Weight Fish (g)	Dose (mL)	Fish Weight (g)	Dose (mL)
300	0.06	500	0.1
310	0.062	510	0.102
320	0.064	520	0.104
330	0.066	530	0.106
340	0.068	540	0.108
350	0.07	550	0.11
360	0.072	560	0.112
370	0.074	570	0.114
380	0.076	580	0.116
390	0.078	590	0.118
400	0.08	600	0.12
410	0.082	610	0.122
420	0.084	620	0.124
430	0.086	630	0.126
440	0.088	640	0.128
450	0.09	650	0.13
460	0.092	660	0.132
470	0.094	670	0.134
480	0.096	680	0.136
490	0.098	690	0.138

11.10 Dose table; LHRHa, intramuscular pellets

Table 11 - 4 Pellet weight required to administer lutenising hormone releasing hormone at a dose of 10µg LHRHa per Kg bwt (g). Doses based on preparation of 1mg LHRHa in 4000mg pellet mix to give pellets of required dose, administered at 4mg per 100g fish weight

Fish weight (g)	Pellet (mg)	Fish weight (g)	Pellet (mg)
300	12	500	20
310	12.4	510	20.4
320	12.8	520	20.8
330	13.2	530	21.2
340	13.6	540	21.6
350	14	550	22
360	14.4	560	22.4
370	14.8	570	22.8
380	15.2	580	23.2
390	15.6	590	23.6
400	16	600	24
410	16.4	610	24.4
420	16.8	620	24.8
430	17.2	630	25.2
440	17.6	640	25.6
450	18	650	26
460	18.4	660	26.4
470	18.8	670	26.8
480	19.2	680	27.2
490	19.6	690	27.6

11.11 Data sheets

11.11.1 Sample broodstock feed sheet

Table 11 - 5 Sample broodstock feed sheet

Broodstock feed sheets				
Date	am/pm	Fresh feed (g)	Pellets (g)	Comments

11.11.2 Sample broodstock mortality sheet

Table 11 - 6 Sample broodstock mortality sheet

Broodstock mortality sheet				
Date	Tank no.	Tag no.	Male /female	Symptoms / comments

11.11.3 Sample water quality sheet

Table 11 - 7 Sample water quality sheet

Water quality sheet for broodstock shed					
Date	Water exchange	Ammonium (mg/L NH_4)	Salinity (‰)	DO_2 (mg/L)	Temperature (°C)

11.11.4 Sample egg collection data sheet

Table 11 - 8 Sample egg collection data sheet

Egg collection data sheet																	
		Fertilised eggs							Unfertilised eggs								
Date	Tank no.	1	2	3	Mean	Sample vol. (mL)	Total vol. (mL)	Total no.	1	2	3	Mean	Sample vol. (mL)	Total vol. (mL)	Total no.	Total no. eggs	% fert.

11.11.5 Sample larval hatching sheet

Table 11 - 9 Sample larval hatching sheet

Larval hatching sheet								
			Sample					
Date	Incubation tank no.	No. of eggs stocked	1	2	3	Average No.	Total no.	% hatch

11.11.6 Sample larval rearing operations sheet

Table 11 - 10 Sample larval rearing operation sheet

Tank number..... Hatch Date..... No. of larvae stocked.....

Date	DPH	Temp (°C)	Light/ dark (hr)	Algae added (L)	Rotfers ($x10^6$)	*Artemia* added (g)	Crumble/ pellet added (g)	Estimated mortality	Other (tank operations etc.)

11.11.7 Sample larval rearing development sheet

Table 11 - 11 Sample larval development sheet

Larval rearing development sheet Hatch:					Date:				Days Post	
Tank no:	Sample no.									
Developmental stage	1	2	3	4	5	6	7	8	9	10
Standard length (mm)										
Yolk sac present										
Eyes pigmented										
Mouth open										
Gut open										
Feeding (y/n)										
Swim bladder (present/inflated)										
Notochord flexion										
Fin development										
Comments										

11.11.8 Sample rotifer feeding records

Table 11 - 12 Sample rotifer culture records

Rotifer feeding records							Tank No…….		
Date	Rotifer (no./mL)	Egg ratio	Tank vol. rotifers	Total no. (mill.)	Yeast (g)		Algae		Tank operations
					am	pm	am	pm	

11.12 Broodstock feeds review

Prior to and during the first spawning season (1998), the KGW broodstock diet at SAASC consisted of cockles (*Donax deltoides*), trawl squid (*nototodarus gouldi*) and a commercial Barramundi pellet. Fertilised eggs produced during that spawning period were of low quality and did not persist to first feeding, indicating that the broodstock nutrition was not adequate.

Several studies have identified a strong relationship between the nutritional composition of broodstock feed and egg quality and quantity (Watanabe *et al* 1984a; Watanabe 1985; Watanabe *et al* 1991a,b; Verakuniriya *et al* 1996; Izquierdo *et al* 2001). Unprocessed fish products are often avoided as they rarely provide adequate levels of nutrients required by broodstock and increases the risk of disease transmission (Bruce *et al*, 1999; Izquierdo *et al* 2001). Pelleted broodstock diets are generally preferred as they allow greater control over the composition of feed, reduce the risk of disease introduction and in a lot of cases reduce costs, particularly labour required to prepare unprocessed products and storage and handling.

To date, no studies on the nutritional requirements of KGW have been conducted. After a review of current scientific literature a KGW broodstock pellet was formulated to include ingredients which had demonstrated benefits to egg and larval quality for marine finfish. The pellet diet was fed year round and all supplementary fresh feeds ceased, substantially reducing labour and feed costs.

Eggs produced during subsequent spawning periods were of higher quality and the percentage of larvae surviving to first feeding increased. The improvement in the quality of eggs and larvae indicate the importance of broodstock nutrition to successive progeny. However, to achieve an optimum broodstock diet for KGW further nutritional studies would be required.

Below is an overview of the essential components in marine finfish broodstock diets, which are thought to play a role in reproduction.

11.12.1 Vitamins

Vitamin E

It is widely accepted that the levels of Vitamin E (tocopherol) in broodstock diets affect reproduction in marine finfish (Kinumaki *et al* 1972; Watanabe and Takashima, 1977; Watanabe 1985, 1990; Watanabe *et al* 1991b; Verakunpiriya *et al* 1996; Fernandez-Palacios *et al* 1997; Izquierdo *et al*, 2001). It is not known yet exactly what role vitamin E plays in reproduction. Some studies suggest that vitamin E acts as an antioxidant, preventing the reactive breakdown of other desirable molecules, especially polyunsaturated lipids (Verakunpiriya *et al* 1996).

Studies have shown the level of vitamin E in the tissues of broodstock reduces after spawning with a high concentration of vitamin E in the eggs, suggesting that vitamin E plays some sort of a physiological function related to reproduction particularly spawning, fertilisation and hatching (Watanabe 1985). Other studies on carp (*Cyprinus carpio*), looking at the effect of vitamin E levels in diets, have shown that broodstock fed diets low in vitamin E have gonads reduced in weight. Broodstock with deficient ovaries failed to accumulate yolk-granules or yolk-vesicles in developing oocytes, indicating that vitamin E plays a more direct role in oocyte development (Watanabe and Takashima 1977).

A lack of vitamin E in the diet has also been shown to inhibit broodstock from spawning, reduce fertilisation and hatch rates and produce larvae with increased mortality rates (Takeuchi *et al* 1981a; Watanabe, 1990). This suggests that vitamin E plays a pivotal role in reproduction and in achieving high quality offspring.

Like most dietary requirements optimal levels of vitamin E varies between species (Izquierdo *et al* 2001). In gilthead seabream (*Sparus auratus*) the percentage of abnormal eggs was significantly reduced when the dietary level of vitamin E was increased from 22 to 125mg/kg (Fernandez-Palacios *et al* 1997). In red seabream increased levels of dietary vitamin E (up to 200mg/kg) resulted in an increased percentage of normal larvae from 33.4% to 72.4% (Watanabe *et al* 1991b). However, it is hard to determine the level of vitamin E in isolation as it is related to the level and type of lipid in the diet. In general broodstock diets contain higher levels than is required in growout diets.

Further studies are needed to determine the precise role of vitamin E in reproduction of marine finfish and determine the optimum level of vitamin E in broodstock diets for a variety of species.

Vitamin A

Little is known about the nutritional benefits of dietary vitamin A during gonadal maturation and spawning. It is however, considered to play an important role in reproduction (Verakunipiriya *et al* 1996). Vitamin A plays a pivotal role in bone development, retina formation and differentiation of immune cells as such, it is considered very important for embryo and larval development (Izquierdo *et al* 2001).

Further studies are required to explore the benefits of vitamin A in the inclusion of broodstock diets and the requirements of dietary vitamin A for different species.

Vitamin B

Very little information is available on the effect of dietary vitamin B in fish reproduction. It is thought that vitamin B_1 (thiamine) needs to be included as a component of broodstock nutrition as there is evidence to suggest it is important for normal embryo and larval development at least in Salmonids (Izquierdo *et al* 2001).

The inclusion of dietary vitamin B_6 into broodstock diets should also be considered due to the important role it plays in the synthesis of steroid hormones and folic acid (Izquierdo *et al* 2001). It is suggested that a deficiency in vitamin B_6 may effect the hatchability of eggs as a result of reduced cell division due to impaired synthesis of DNA and RNA (Halver 1989 as cited in Izquierdo *et al* 2001).

Further research is needed to define the effects of the vitamin B group on fish reproduction and the quality of subsequent progeny.

Vitamin C

Vitamin C (ascorbic acid) is considered to play an important role in vitellogenesis, maturation and subsequent egg quality from finfish broodstock. The exact role vitamin C plays in the reproduction

process is not known.

Studies have found that the level of vitamin C in gonads changes throughout the reproductive cycle, with an increase during early vitellogenesis (Seymour, 1981; Sandnes and Brekkan, 1981; Sandnes, 1984 as cited in Kjorsvik *et al*, 1990). Research into the effect of varied levels of vitamin C (0, 50, 500 mg/g) in cod broodstock diets showed that diets with the highest inclusion of vitamin C had the highest incorporation of vitamin C in their gonads (between 300-700 mg/g). The inclusion of supplementary vitamin C in broodstock diets (115 mg/kg after processing and drying has been shown to improve hatching rate of eggs in rainbow trout (Kjorsvik *et al*, 1990; Emata *et al*, 2000) and a positive correlation was found between the content of vitamin C in broodstock diets and eggs (Sandnes *et al*, 1984). The exact role of vitamin C in improving hatch rates was not identified.

It has been suggested that the important role vitamin C plays in the synthesis of collagen during embryo development may be related to improved egg quality and survival of progeny (Izquierdo *et al*, 2001).

Other studies suggest that in males it is the anti-oxidant properties of vitamin C (and vitamin E) that provide an important protective role for the sperm cells during spermatogenesis and until fertilisation. It is thought that it does this by reducing the risk of lipid peroxidation which is determined by sperm motility (Izquierdo *et al*, 2001), consequently improving fertilisation rates.

While the exact role of vitamin C in the reproduction process is not known, it seems likely that it is playing an important role in maturation and subsequent egg quality of finfish broodstock.

The level of vitamin C required varies between species and the developmental stage of the fish. Further studies are needed to identify the level of dietary vitamin C required for broodstock of different species.

Vitamin D

Few studies considered the nutritional significance of vitamin D. Research on rainbow trout suggests that while no actual level of vitamin D required in a broodstock diet could be determined, the level was lower than that of Vitamin A and E in rainbow trout (Kinumaki *et al* 1972 as cited in Watanabe 1985).

11.12.2 Pigments

Several studies have considered the importance of carotenoid pigments in broodstock diets with respect to broodstock reproduction and subsequent egg quality.

Results of studies (Bromage 1993; Watanabe *et al* 1984b; Watanabe 1985, Kjorsvik *et al* 1990 and Watanabe *et al* 1991a,b) suggest that carotenoids play a significant role in broodstock diets of finfish in general, improving the overall viability of eggs and larvae. To date it is not know in what way pigments enhance reproduction.

Carotenoids constitute one of the most important pigment classes in fish with a wide variety of functions including protection from adverse lighting conditions, a precursor for synthesis of vitamin A source and chemotaxis of spermatazoa. They also have antioxidant functions with a strong ability for quenching singlet oxygen (Watanabe *et al* 1991b; Bromage and Roberts 1995; Izquierdo *et al* 2001). Studies by Watanabe *et al* 1984b (as cited in Watanabe 1985) suggest that it may be the anti-oxidant properties of carotenoids that fulfill some physiological function rather than a requirement for the pigment itself.

The beneficial effect of pigments in broodstock diets is supported in further studies by Watanabe *et al* 1991a on red seabream where results showed that feeding frozen raw krill increased productivity (rate of buoyant eggs and hatchability). Frozen raw krill was broken down to investigate what component of frozen raw krill was having a positive effect. The results indicated that the effective components of krill are in the oil fraction. When further broken down both the polar and non-polar lipid fractions increased the rate of buoyant eggs and hatchability. In the non-polar lipids it is suggested that it is the properties of another pigment astaxanthin (i.e. free radical scavenger) that were one of the effective components in frozen raw krill, enhancing the quality of eggs.

The effect of pigments included in red seabream, (*Pagrus major*), broodstock diets were evident within a matter of hours making them a useful supplement during spawning periods (Watanabe *et al* 1984b). The level of pigments required in broodstock diets is not known and is thought to vary between species and maybe dependent on the quantities of other components within the diet.

11.12.3 Protein

Protein: Energy

The ratio of protein to lipid (energy) included in broodstock diets is thought to effect the quality of eggs produced.

Studies on red seabream examined the effect of low-protein, high calories diets on the reproductive performance of red seabream. Results indicated that the number of eggs, the percentage buoyancy, number of egg abnormalities, hatchability and larval deformity were adversely affected in broodstock fed the low-protein high calorie diet (33% protein; 16% lipid) in comparison to the control diet (45% protein; 10% lipid). This suggests that the protein content of diets strongly affects the reproductive performance of red seabream broodstock (Watanabe *et al* 1984a). Similarly Bromage 1993 suggests that optimum protein levels for gilthead seabream *(Sparus auratus)* are 55% protein based on squid meal, containing 10-15% marine type lipids. This notion is supported in studies in seabass *(Dicentrarchus labrax),* where a reduction in dietary protein levels from 51% to 34% and an increase in carbohydrate levels to 32% reduced egg viability (Cerda *et al* 1994b).

This is in contrast to studies conducted on rainbow trout where Watanabe *et al* 1984d, found that growth rate, feed efficiency and egg quality (where total numbers of eggs produced; average egg diameter; eyed rate and total hatch were used as egg quality parameters) were comparable between diets containing 36% protein, 18% lipid and 46% protein, 15% lipid. Takeuchi *et al* 1981b (as cited in Watanabe 1985) found similar results in rainbow trout fingerlings, suggesting that for both fingerlings and broodstock of rainbow trout a low protein, high energy level diet is as effective as conventional diets containing higher levels of protein.

These studies indicate that the optimum protein to energy ratio may be species and age specific.

Protein Source

Fish meal is generally considered as an acceptable protein source for broodstock diets in a range of finfish species. Fish meal can vary in digestibility and amino acid balance. The addition of other protein sources may improve performance of broodstock.

Studies by Watanabe *et al* (1984a); Watanabe *et al* 1991b; Fernandez-Palacios *et al*, 1997) examined the effect of different protein sources in broodstock diets to the reproductive performance

of finfish. Cuttlefish meal and squid meal were found to increase growth rates (broodstock), total number of eggs produced, percentage buoyancy and hatch rates, therefore considering cuttlefish and squid meal as a superior protein source. These studies considered that it was the fat-insoluble fraction of the meal that was having the effect.

It has also been suggested that the improved performance of broodstock fed diets with squid or cuttlefish meal may be due to the increased digestibility of squid and cuttlefish meal (Fernandez-Palacios *et al*, 1997) and the lipid fraction (Mourente *et al*, 1989). Squid and cuttlefish meal may also indirectly improve performance by acting as an attractant, increasing the amount consumed by fish and thereby increasing their nutrition.

11.12.4 Lipids

One of the major problems with formulating artificial diets for marine finfish is satisfying their lipid requirements (Bruce *et al*, 1999). The level of essential fatty acids in broodstock diets, in particular acarchidonic acid (20:4 n-6, AA), eicosapentaenoic acid (20:5 n-3; EPA) and docasahexaenoic acid (22:6 n-3; DHA) is a major factor in the reproductive performance of both male and female broodstock (Bromage and Roberts, 1995; Izquierdo *et al* 2001). It is important to note that it is not only the total lipid content in diets that can effect the reproductive performance of marine finfish, but also the ratio of DHA:EPA and (n-3):(n-6) (Bromage and Roberts, 1995; Bruce *et al*, 1999).

Fish cells contain high levels of the n-3 highly unsaturated fatty acid (HUFA) series, EPA and DHA. n-3 HUFA have a generalised role in the cell membranes of fish and in some species (eg. halibut) they are considered a major energy source during early embryonic development (Falk-Peterson *et al*, 1989; Fernandez-Palacios *et al*, 1995; Bromage and Roberts, 1995).

AA while not contained in high levels in fish cells, also has a vital role. It is the main precursor to eicosanoids (biologically active compounds) which are important in a wide range of physiological roles including the reproductive system (Bruce *et al*, 1999).

Marine teleosts unlike freshwater teleosts, lack the ability to synthesise their own essential long-chain HUFA and it is therefore imperative that they obtain sufficient levels of DHA, EPA and AA from their diet (Bruce *et al*, 1999). Numerous studies have shown that the level of essential fatty acids in the diet is reflected in the quality of eggs (Watanabe *et al* 1984a; Fernandez-Palacios *et al*, 1995; Navas *et al*, 1996; Bruce *et al* 1999;).

Results of studies by Watanabe *et al* 1984a on red seabream, fed broodstock diets deficient in fatty acids indicated that a lack of EFA in broodstock diets did not effect fecundity (the numbers of eggs produced), but it did cause a reduction in the number of buoyant eggs produced and increased the percentage of abnormalities (up to 93.7% compared with 30.7% in the control group) resulting in lower numbers of viable larvae produced. Similar results were found in gilthead seabream where fish oil was used rather than soybean oil to increase the level of n-3 HUFA in diets. The quality and survival of resultant larvae was increased (Tandler *et al* 1995 as cited in Izquierdo *et al* 2001). Other studies (Fernandez-Palacios *et al* 1995 and Navas *et al* 1996) support these findings and highlight the importance of n-3 HUFA as a component in broodstock diets.

It is important to note that the inclusion of n-3 HUFA levels that are too high, can have a detrimental effect on reproduction and subsequent progeny. Excessive levels of n-3 HUFA in broodstock diets (>2% dry weight) fed to gilthead seabream (*Sparus auratus*) resulted in yolk sac hypertrophy and a decrease in larval survival (Fernandez-Palacios *et al* 1995).

Again the required levels of lipids and ratio of lipids in diets varies within and between species as the fatty acid composition of fish egg lipids is related to species and stock differences as well as the broodstock diet (Pickova *et al*, 1997 as cited in Izquierdo *et al* 2001). The essential fatty acid requirements for sparids broodstock are between 1.5 and 2% n-3 HUFA in the diet (Watanabe *et al* 1984a, b, c; Fernandez-Palacios *et al*, 1995) whereas the optimal level of n-3 HUFA in the diet for salmonids is approximately 1% (Watanabe, 1990 as cited in Izquierdo *et al* 2001). Further studies are required to identify optimal levels of lipids required in a range of species.

11.13 Licensing

To establish land based farms in South Australia, you must apply to Primary Industries Resource South Australia (PIRSA) Aquaculture licensing at 14th floor 25 Grenfell St, Adelaide, Adelaide, SA, 5001. Phone: (08) 8226 2311. Applications are also available on the website www.pirsa.gov.au.

To hold fish in state waters a section 50 permit is required which costs $100.

For both land and sea based farms developmental approval is required as per the development act 1993 (planning consent). For further information contact planning SA on (08) 8207 2000 or on their website at www.planning.sa.gov.au.

You can contact a professional fisherman who will collect broodstock from the wild, however, if that is not possible you can apply in writing to the deposit of fisheries to obtain a section 59 permit at a cost of $100. This is required each time broodstock are sourced from the wild.

11.14 References

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