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Gamete quality and spawning in captive Murray cod broodstock

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Gamete quality and spawning in captive Murray cod broodstock

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Executive Summary

The quality of the eggs and sperm (gametes) produced by broodstock fish greatly influences the amount and quality of the next generation of fish.

Artificial breeding techniques have been developed for Murray cod (*Maccullochella peelii*), but studies have shown that while intensively reared, tank-matured Murray cod readily spawn, the hatch rates of spawned eggs were very low compared to fish spawning naturally in ponds. In order to maximise a breeding program for Murray cod and to provide selectively bred fish to the developing Murray cod farming industry, the hatch rates of captive bred fish need to be increased.

Evaluation of gamete quality parameters can assist in improving the reproductive output of captive broodstock. Despite extensive work on breeding Murray cod in captivity over 40 years, there has been no detailed study on the quality of ova and sperm, and associated affects on spawning.

The objective of the current study was to investigate factors effecting gamete quality in captive Murray cod broodstock.

The growth of male broodstock was slightly faster than females in controlled hatchery conditions. Rearing broodstock in captivity under controlled hatchery conditions reduced the time to maturation. Maturity was reached at 3 years of age for many males and at 4 years for most females. There were more females than males in the broodstock population (F:M 1.91:1), suggesting that conditions in the hatchery during early development of larvae influenced sex differentiation. Hermaphrodism was confirmed in two broodstock.

A comparison between pond-matured and tank-matured broodstock showed considerable differences in hatch rates. Apart from environmental differences in these culture systems, there were significant differences in the chemical composition of spawned eggs indicating broodstock nutrition may play an important role in gamete quality.

A range of gamete quality parameters were measured during the study including, broodstock age, size and condition, oocyte developmental stage, morphology and appearance, egg morphology and appearance, relative fecundity, milt volume, sperm morphology, density, motility and velocity, fertilisation rate, hatch rate and larval survival rate.

There were considerable variations in oocyte and egg characteristics. In the days following fertilisation, the percent of eggs that remained viable (had not died) declined at various rates with most mortalities occurring in the first three days following fertilisation. Critical stages of embryonic development affecting survival may occur during this period.

There was a high incidence of sperm with hooked flagella, but the cause of this and its affect on sperm viability is unknown. This report described the density of Murray cod sperm for the first time, but further research is now required to identify sperm-to-egg ratios for maximising fertilisation of Murray cod eggs. Improved understanding of sperm motility in the minutes following activation resulted in changes to fertilisation methods during spawning of Murray cod.

Freezing sperm in liquid nitrogen greatly reduced sperm motility, but other characteristics, such as velocity, were unaffected. Fertilisation trials using thawed sperm resulted in hatched larvae, but hatch rates were considerably lower than for eggs fertilised with fresh sperm. These results suggest that cryopreservation techniques need to be refined to improve sperm quality (motility). Refinement of sperm cryopreservation methods will improve management of breeding programs for aquaculture, stock enhancement and conservation. A sperm bank has been established at Snobs Creek with frozen samples of sperm from selected Murray cod broodstock, as well as other species (Macquarie perch and trout cod).

A diet specifically formulated for Murray cod broodstock improved the condition of females and slightly increased relative fecundity compared to broodstock fed a non-specific commercial grow-out diet. Fertilisation rates and hatch rates were not improved by this diet, suggesting other factors may be involved in gamete quality.

During the project, two females ovulated without intervention with hormone treatments resulting in high hatch rates. This suggests that intensively reared broodstock have the potential to mature and ovulate unassisted. Continued breeding of Murray cod in captivity using domesticated lines over multiple generations may see improvements in spawning.

Analysis of information for 1,170 spawning events identified a number of significant correlations between gamete quality indices, spawning evaluation parameters, and measures of spawning. Hatch rate was positively correlated with fertilisation rate, week of spawning season, and volume of milt used to fertilise eggs, and was negatively correlated with the proportion of stripped eggs that were coalesced and had cloudy or dirt marks present, both of which may be characteristic of over ripening.

Information presented in this report will assist in improving hatchery production not just of Murray cod but other Maccullochella spp. (trout cod, eastern freshwater cod and Mary River cod) that are produced for stock enhancement purposes.

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Introduction

Farming of fish

Aquaculture is the farming of aquatic organisms such as fish, crustaceans, molluscs and aquatic plants.

As aquaculture operations become more sophisticated, selective breeding programs have been developed to improve and enhance production through selection of stock with traits of value for farmers, markets and consumers (Gjedrem 2005, Gjedrem 2012).

Murray cod (*Maccullochella peelii*) is a freshwater fish that is endemic to the Murray-Darling River system. Murray cod are highly valued for recreational, commercial and conservation purposes. It is highly sought after as a table fish.

Murray cod aquaculture

During the late 1970's and early 1980's breeding techniques for Murray cod were established and, as a result, many hundreds of thousands of fingerling Murray cod are now produced in hatcheries annually. These juveniles are used for stocking public and private waterways for both recreational and conservation purposes (Ingram and De Silva 2004).

More recently there has been considerable interest (from both producers and markets) in the grow-out of Murray cod to satisfy a significant domestic and export demand for human consumption (Ingram *et al.* 2005a).

Farming Murray cod to produce high-quality table fish for domestic and export markets is a rapidly emerging agri-business sector in south eastern Australia.

Captive breeding

Traditionally, Murray cod bred in captivity are held in earthen ponds and allowed to spawn unassisted (Rowland 1983, Cadwallader and Gooley 1985). This method of reproduction provides little control over which fish mate, and may effect the genetic structure of progeny as not all broodstock contribute equally to the spawning (Rourke et al. 2009).

Effective breeding programs require the active management of mating, which can be achieved by using artificial spawning methods where specific females and males can be crossed. To fully realise the potential of a selective breeding program for Murray cod, rigorous control of mating needs to be undertaken (Ingram et al. 2007, Ingram et al. 2008).

Selective breeding

Artificial spawning techniques for finfish typically involve the use of hormone to induce spawning, followed by hand stripping of gametes (De Silva et al. 2007). Artificial spawning techniques have been developed for Murray cod (Rowland 1988).

While Murray cod can be induced to spawn in captivity under artificial conditions, the hatch rates of stripped eggs are very low compared to fish spawning naturally in ponds (Ingram et al. 2007, Ingram et al. 2008).

Quality of eggs and sperm

The quality of the eggs and sperm (gametes) produced by broodstock greatly influences seedstock production for both commercial aquaculture operation and for recreational fisheries stock enhancement (Brooks et al. 1997, Rurangwa et al. 2004, Bobe and Labbé 2009, Cabrita et al. 2009, Lahnsteiner et al. 2009, Fauvel et al. 2010).

Gamete quality is influenced by a number of factors, which include:

- Environmental conditions in which the broodstock fish are maintained (e.g. temperature, water quality or photoperiod)
- Behavioural factors where broodstock are maintained in circumstances where there is constant interaction with other fish
- Husbandry practices (cleaning tanks, handling fish and spawning techniques)
- Diet (see Bromage and Roberts 1995, Izquierdo et al. 2001, Soso et al. 2008, Mylonas et al. 2010, Callan et al. 2012).

Improving performance

In order to maximise the effectiveness of a Murray cod selective breeding program, the hatch rates of eggs produced from captive bred fish need to be increased.

Despite extensive work on breeding Murray cod in captivity over 40 years (e.g. Wyse 1973, Rowland 1983, Cadwallader and Gooley 1985,

Rowland 1988, Newman et al. 2008a, Newman et al. 2008b, Newman et al. 2010), there has been no detailed study on how the quality of eggs and sperm affect hatch rates and the quality of the next generation.

Evaluation of the factors that affect the quality of gametes produced by Murray cod will assist in improving the reproductive output of captive broodstock.

Objectives

The objective of the current study was to investigate factors effecting gamete quality in captive Murray cod broodstock.

Project Design and Methods

General methods

Facilities

All experiments were conducted in hatchery facilities located at the Department of Primary industries facilities at Snobs Creek, (Eildon) between July 2008 and January 2012.

Broodstock

Broodstock were maintained in recirculating aquaculture systems (RAS) located in either the Native Fish Building (Hesy) (Figure 1a), or the Greenhouse Facility (RAS1 and RAS2) (Figure 1b). Tank facilities ranged from 800 to 2,500 L in volume.

Operation and maintenance of the systems is described in the greenhouse operations manual (Anon 2010). Briefly, water was supplied to the facilities from either Snobs Creek or the Goulburn River. Incoming water was filtered, treated (UV and Ozone sterilisation) and heated to maintain a constant desired temperature. Water flow through the tanks ranged from 1–20 L/min (depending on tank size and fish density). All tanks were aerated. The quality of the water was tested periodically ranging from weekly to monthly. Photoperiod in the Hesy system was maintained by a Cfish Electronic Photoperiod controller System (Kingston, Tasmania), which was set to a natural regime for the region. The RAS1 and RAS2 systems received ambient light. Water temperature was controlled by a gas boiler and chiller unit, and the regime mimicked a typical annual natural ambient profile.

Previous studies have shown that Murray cod cease spawning once water temperature reaches 20 °C (Rowland 1983, Cadwallader and Gooley 1985). Therefore, once the water temperature reached 18-19 °C, it was maintained at this level for the duration of the season to ensure that broodstock stayed in spawning condition for as long as possible.

All tanks were cleaned regularly. Uneaten food and faecal material was removed and the sides and floor scrubbed clean. Tanks were also purged regularly.

Egg incubation and hatching

Egg incubation and hatching was undertaken in specially designed vertical incubators (Figure 1c), 100 L troughs fitted with egg incubation baskets (Figure 1d) or in 160 L tanks (Figure 1e and f) that received a constant flow of fresh filtered and heated water. Larvae were reared in either 100 L troughs or 160 L tanks.

Experiments were conducted under the DPI Fish Animal Ethics Committee (AEC) Application Sept08 0050 Aquaculture Futures Initiative.

Broodstock management

All broodstock used in experiments were first generation fish that were obtained as larvae from Murray cod hatcheries in 2003 or 2005. These fish were obtained as part of a project to develop a genetic selective breeding program for the species (Ingram et al. 2007, Ho et al. 2008, Ingram et al. 2008). These fish represented progeny from 42 different families. All fish were implanted with a passive integrated transponder (microchip) for identification purposes and to ensure that siblings were not mated together.

Broodstock were randomly assigned to fibreglass tanks at similar densities and according to sex. Densities did not exceed 70 kg/m^3.

Since specific diets for Murray cod are not available, commercially available diets formulated for barramundi were fed to the fish. (Ridley or Skretting Brands) (Table 1).

Generally, floating pellets were used to ensure that feeding activity could be monitored. Fish were fed to satiation every one to three days (depending on the water temperature). During 2008 and 2009, diet of broodstock was supplemented with thawed rainbow trout carcasses and fillets.

Handling

Generally broodstock were handled only during the spawning season (October –January). When handled (for measurement, tagging and spawning) fish were anaesthetised using Aqui-S (Aqui-S NZ Ltd.) (0.01–0.1 mL/L, depending on size of fish) Procedures followed DPI Fish AEC SOP 03 – Fish anaesthesia and DPI Fish AEC SOP 21 – General anaesthesia in fish.

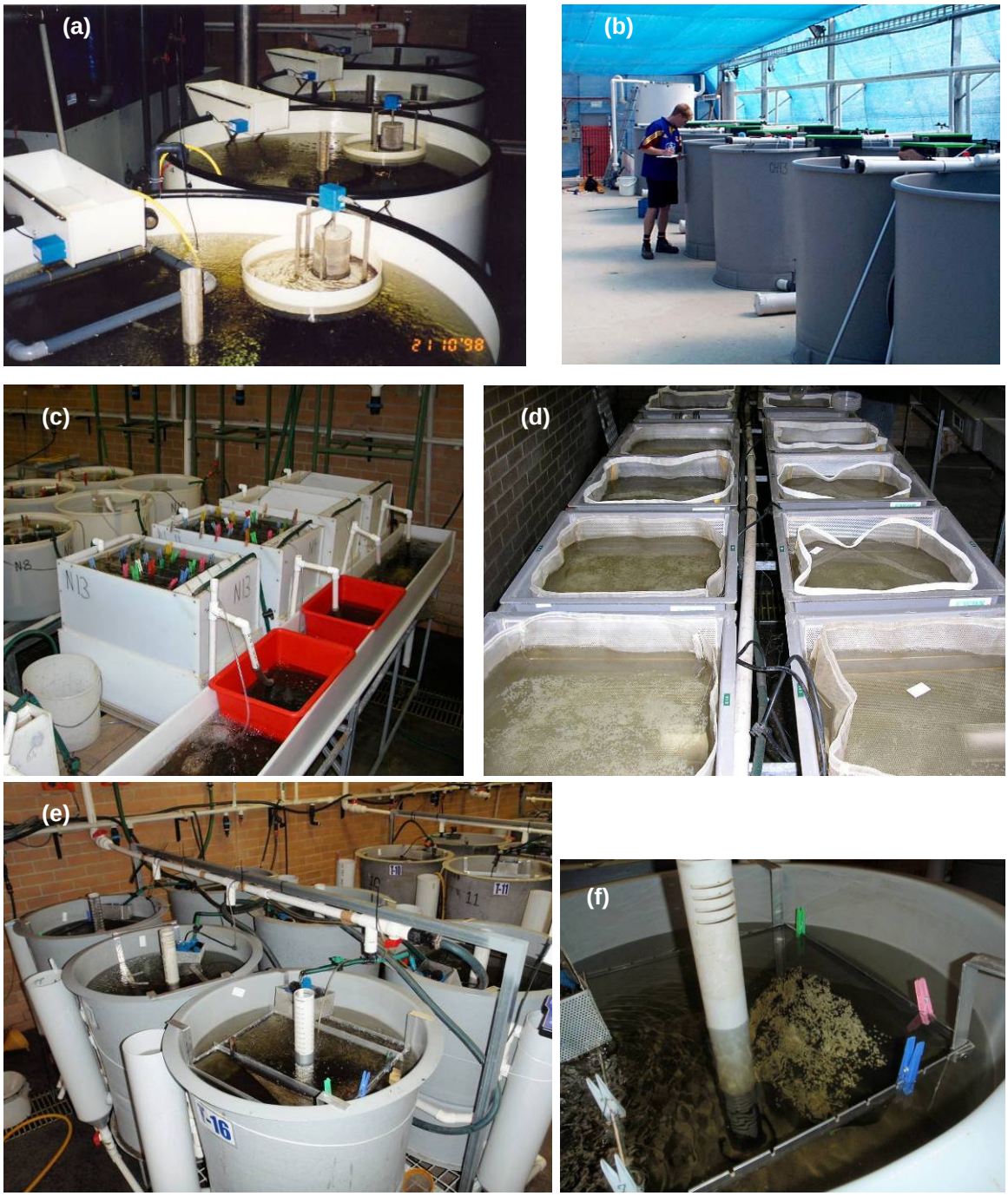

Figure 1. Snobs Creek Hatchery facilities for captive breeding of Murray cod. (a) Hesy recirculating aquaculture system, Native Fish Building. (b) Greenhouse recirculating aquaculture system. (c) Murray cod egg incubators. (d) Ewos trough fitted with egg incubation baskets (e) 160 l tanks used to incubate eggs. (f). Eggs in 160 l tank.

Table 1. Proximate composition of commercial diet and experimental broodstock diet used for feeding tank-reared Murray cod broodstock.

Parameter	Deakin University Broodstock diet* (DU)	Ridley Marine 45/20** (Rid)
Type	Floating	Floating
Size (mm)	20-25	15
Protein (%)	50	45
Lipid (%)	17	20
Fibre (%)		2.5
Ash (%)	5	11.2
NFE (%)	22	16
Gross energy (MJ)	23	21.29

* Detailed description is provided in Appendix II.
** Specifications from feed Manufacturer (http://www.agriproducts.com.au/)

All fish are given a prophylactic salt bath (5–10 g/L for 0.5–1 hour, depending on size) after handling, and then are returned to the culture system.

Disease control treatments (therapeutic and prophylactic) are outlined in DPI Fish AEC SOP 02 – Disease treatment, and we also followed treatments described in Ingram et al. (2005b).

Oocyte sampling and stages of development

Ovary and oocyte developmental stages described in this report are modified from Snobs Creek historical notes, Rowland (1998b), Newman et al. (2007) and Núñez and Duponchelle (2008) (see Appendix I).

Mature females display a distended abdomen and swollen vent (due to the development of mature ovaries). The stage of oocyte maturity was assessed from a sample of oocytes cannulated from the ovaries of anaesthetised fish using a glass catheter (Figure 2). Typically, mature oocytes were about 3–3.5 mm in diameter and were translucent (Figure 3a-d).

If females were immature (i.e. had a high proportion of stage S5 oocytes less than 2 mm in diameter) (Figure 3a and c), or showed signs of oocyte resorption (Figure 3e and f) fish were not injected.

Oocytes were measured and photographed using a dissecting microscope fitted with a calibrated eyepiece micrometer and a C-mount Camera.

Eggs diameters were measured directly with a calibrated eyepiece micrometer or from captured images using Able image Analysis or ImageJ software.

Sperm sampling and analysis

Mature males express milt when pressure is applied to the abdomen (Figure 4). For sperm analysis, a 3 µL sample of milt was collected with a micropipette from running ripe fish. The sampled was mixed with 97 µL of freshwater in an eppendorf tube, then a 20 µL sub-sample of diluted milt was placed in a Hamilton Thorne 2x-cel sperm chamber and examined under high magnification (400x) where image capture was undertaken for sperm analysis. Samples were analysed within 20 seconds of activation in freshwater.

Sperm activity image sequences were captured using an Allied Vision Technologies Marlin F046C digital camera fitted to an Olympus compound microscope fitted with phase contrast objectives.

Sperm analysis was undertaken subjectively (Table 2) and objectively by using the computer-assisted sperm analysis (CASA) plugin software for the National Institutes of Health software ImageJ (http://rsb.info.nih.gov/ij/plugins/casa.html) (Wilson-Leedy and Ingermann 2007). This software was developed primarily for analysis of fish sperm and measures the following parameters:

- Percent motility
  Percent of sperm moving in a manner fitting motility determination parameters.
- Velocity curvilinear (VCL).
  Point to point velocity (total distance travelled) (µm/s).
- Velocity average path (VAP).
  Point to point velocity (µm/s) on a path constructed using a roaming average. The number of points in the roaming average is 1/6th of the frame rate of video used.
- Velocity straight line (VSL).
  Velocity (µm/s) measured using the first point and the average path and the point reached that is furthest from this origin during the measured time period.
- Linearity (LIN).
  VSL/VAP, describes path curvature.
- Wobble (WOB).
  VAP/VCL, describes side to side movement of the sperm head.
- Progression (PROG).
  The average distance of the sperm from its origin on the average path during all frames analysed; division of this variable by VAP yields a description of the average efficiency in terms of the portion of generalized motion that results in movement away from the origin (a perfectly circular path will have a higher efficiency than an erratic path with the same linearity).

Spermatozoa density in collected milt samples was estimated from the captured digital images (see Fauvel et al. 2010) by using the following formula:

$$\text{Spermatozoa } (\mu L) = \frac{\text{Mean No. spermatozoa per image}}{\text{Vol. of image}} \times \text{Milt dilution factor}$$

Where:

Vol. of image = 134.14 x 100.61 x 20 μm = 2.699 x 10^5 μm = 2.699 x 10^{-4} μL.

Milt dilution factor = (Vol. of milt sample (μL) + dilutant added (μL))/ Vol. of milt sample (μL).

When sperm was required for fertilising eggs, milts were collected using a clean dry 5 mL syringe. Milt was collected carefully to avoid contamination with water, urine and faeces (Figure 4). Milt was collected immediately prior to fertilising eggs.

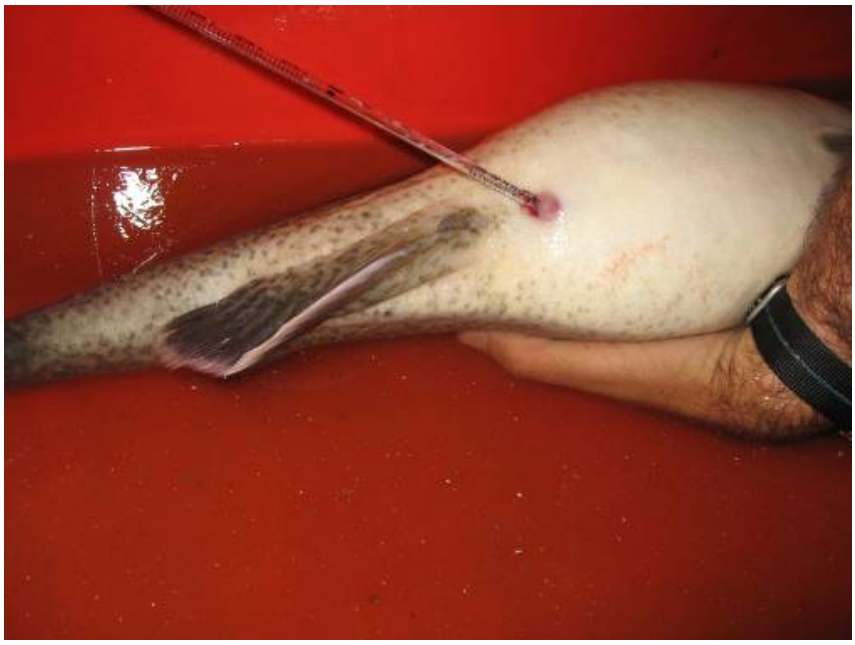

Figure 2. Cannulation of a sedated Murray cod to obtain a sample of oocytes from the ovary.

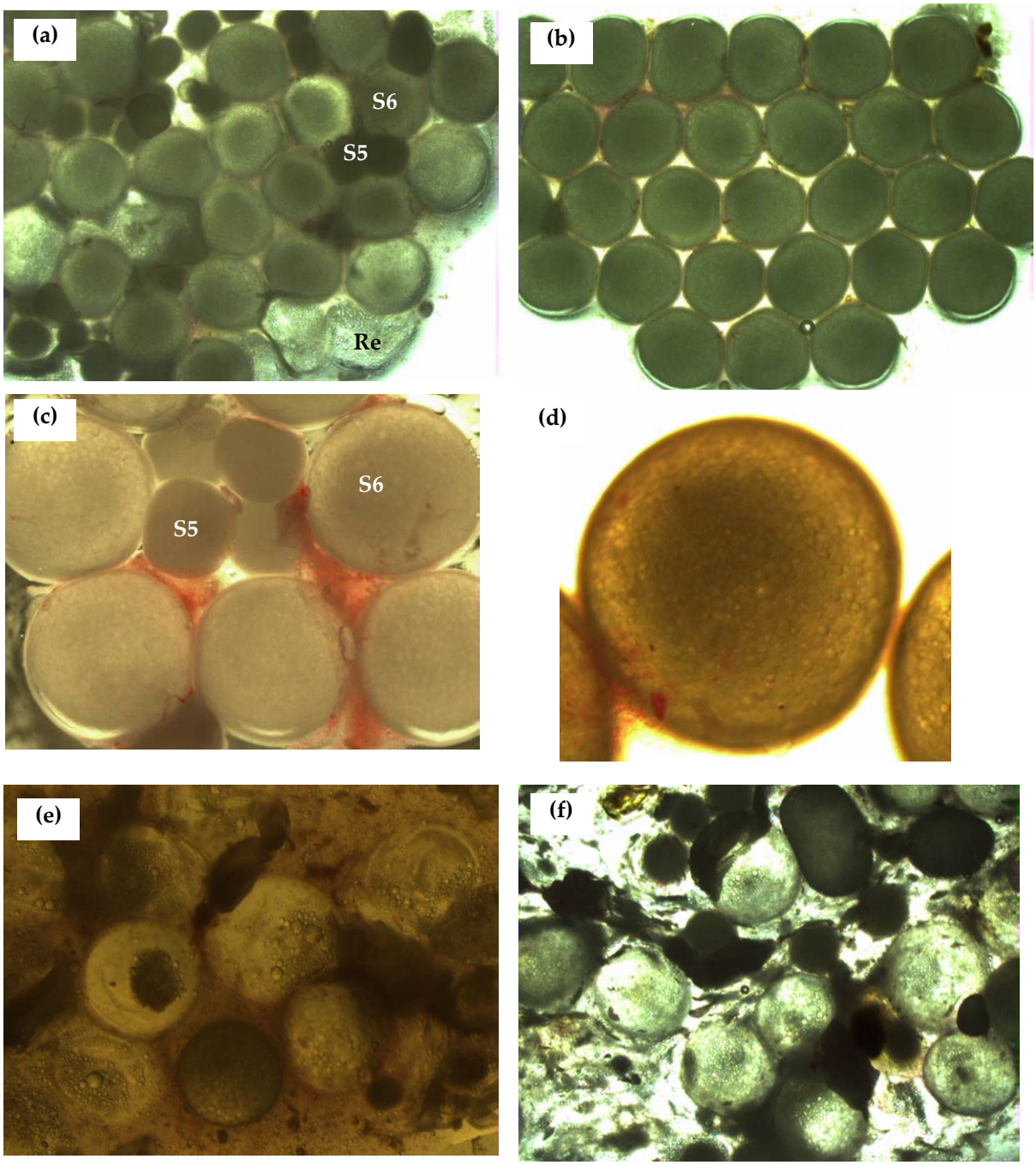

Figure 3. Oocyte samples from mature Murray cod, pre hormone injection. (a) Sample containing S5, S6 and Resorbing (Re) oocytes. (b) Sample of S6 oocytes. (c) Sample of S5 and S6 oocytes. (d) S6 oocyte. (e) Resorbing oocyte sample. (f) Resorbing oocyte sample.

Figure 4. Collecting milt from a sedated male Murray cod.

Table 2. Subjective measures of assessment for Murray cod sperm.

Milt Volume	Sperm Consistency	Sperm Activity
0 = Nil	**1** = Very watery	**0** = Nil activity
1 = Small amount (drops)	**2** = Watery/milky	**1** = Poor ('jiggly". <10% motile)
2 = "Good" (up to 1mL)	**3** = Milky	**2** = Fair(10-50% motile)
3 = "Lots" (>1mL)	**4** = Thick creamy-white	**3** = Good (>50% motile)
		4 = Excellent (100% motile in swirling masses)

Statistical analyses

Unless specified, the SAS General Linear Models Procedure and Tukey's Multiple Range Test (SAS Institute Inc.) were used to test for a significant difference between treatments. Prior to analysis, data sets that were identified as heterogeneous by using Cochran's Test for homogeneity were transformed (log transformation unless specified).

Induced spawning

Methods used to spawn Murray cod in the current project more or less followed established methods described in Cadwallader and Gooley (1985), Rowland (1988) and Ingram and Larkin (2000).

Spawning trials commended each season once water temperature in the culture systems exceeded 16 °C. Broodstock were sedated and gamete samples were collected to determine maturation status.

Hormone induction and latency

Mature fish were induced to spawn with a single injection of Human Chorionic gonadotrophin (HCG) (Chorulon®, Intervet Australia Pty Ltd).

Fish were injected into the peritoneal cavity behind the left pelvic fin. Injected fish were placed into a holding tank (1,000–2,000 L) containing water that was aerated and heated to 20–22 °C throughout the latency period (time from injection to stripping).

Females received a dose of 1,000–2,000 international units (iu)/kg while males received a dose of 500–1,500 i.u./kg. Males were not injected if sufficient milt could be expressed.

Ovulation and stripping

Ovulation, which is accompanied by an increase in the redness and swelling of the vent region, usually occurred 46–50 h after injection. When a female ovulated, eggs flowed freely from the vent with only slight pressure to the abdomen.

Ovulated eggs were clear with oil globules, which ranged in size from 40–600 μm in diameter, could be clearly seen clumped together into groups from 14 to in excess of 100 globules (Figure 5a–e). Some batches of ovulated eggs also had dark areas internally (Figure 5f and g).

Fish were anaesthetised and held upside down in a waterbath, with the vent held above water. Gametes (eggs and sperm) were then hand-stripped from them. This method reduces stress and the risk of damage to fish while stripping.

Ovulated eggs were collected into a clean, dry plastic beaker (Figure 6a).

Milt (up to 1 mL), freshly stripped from a male, was then mixed into the eggs by swirling. A small amount (up to 10% by volume) of fertilising solution (0.4% sodium chloride and 0.3% urea) was also added to:

- Facilitate sperm activation
- Facilitate clearing of the micropyle of the egg
- Facilitate mix of eggs and sperm
- Prevent the eggs from sticking together.

After 5–7 min the fertilised eggs were rinsed with fertilising solution then spread in a single layer onto squares of flymesh (Figure 6b). The flymesh was then immersed in an Ewos trough which contained flowing and aerated water (Figure 6d). Upon emersion in freshwater the eggs become adhesive.

Egg incubation and larviculture

Once immersed in freshwater, eggs water-harden and become opaque (Figure 7a, b and c). After 24 h, the flymesh squares with eggs attached were transferred to incubator tanks (Figure 7c–f). At this stage 24 h post fertilisation egg viability was determined.

Eggs were maintained in a constant flow of well-aerated water maintained at a temperature of 18–22 °C. Eggs were treated with formalin (1,000 ppm for 30 minutes) daily until hatching commenced after which the treatment was reduced to 15 minutes (Ingram *et al.* 2005b).

Embryonic development of eggs could not be readily monitored during the incubation period. The opaque shell of the eggs limited observations of the developmental processes occurring within. Although the shell could be removed with fine forceps to view the developing embryo (Figure 7c–e), this was laborious and was not routinely undertaken.

For each spawn, at completion of hatch, the number of larvae was counted (to determine hatch rate). The length of 10–20 larvae were measured using a dissecting microscope fitted with a calibrated eyepiece micrometer. Hatched larvae (Figure 8) were transferred from incubators to clean Ewos troughs that received a constant flow of well-aerated water.

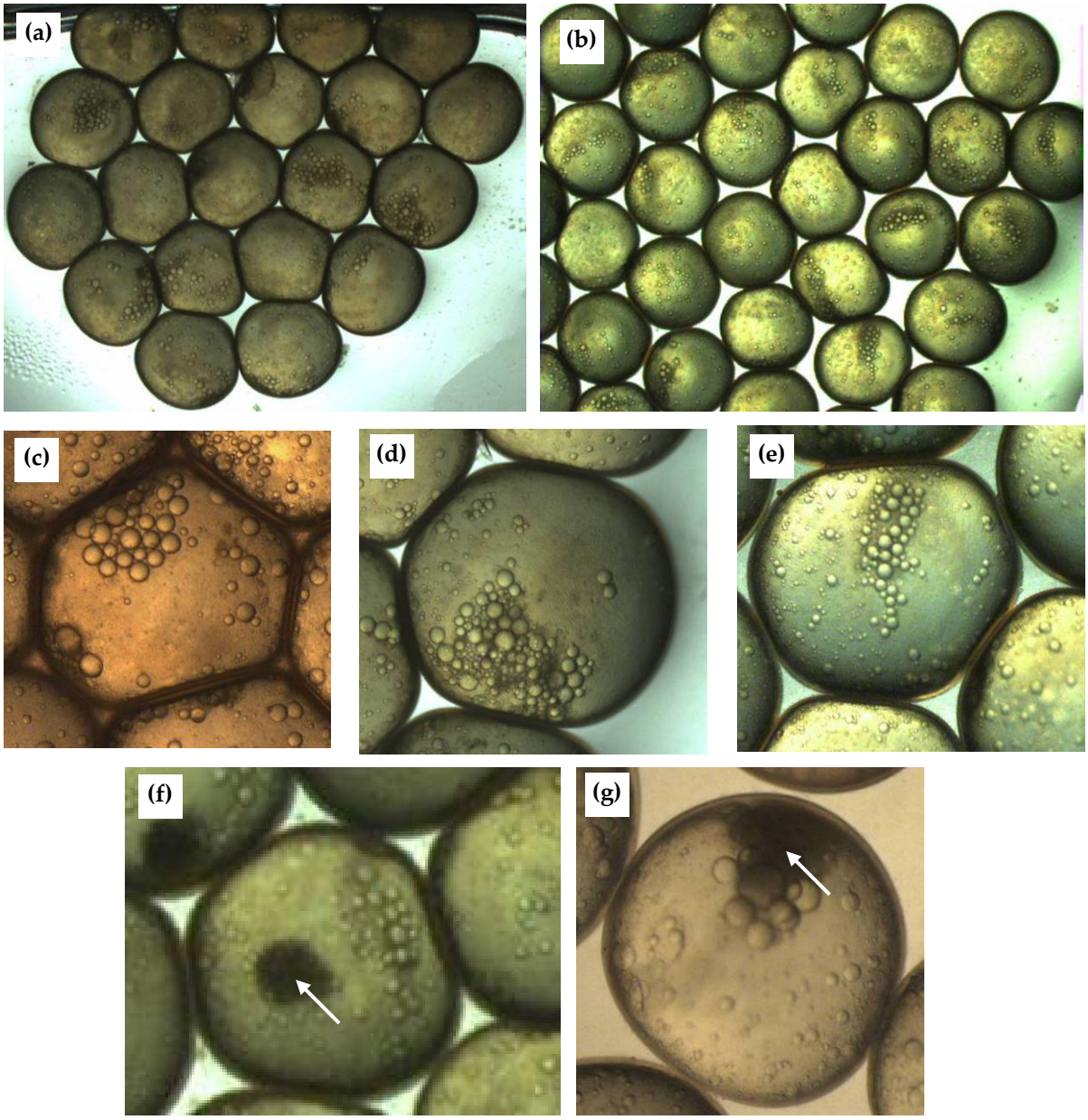

Figure 5. Ovulated Murray cod eggs (a - e) Typical ovulated eggs showing coalesced of oil globules. (f – g) Ovulated eggs with unknown "dark mass" (arrow)

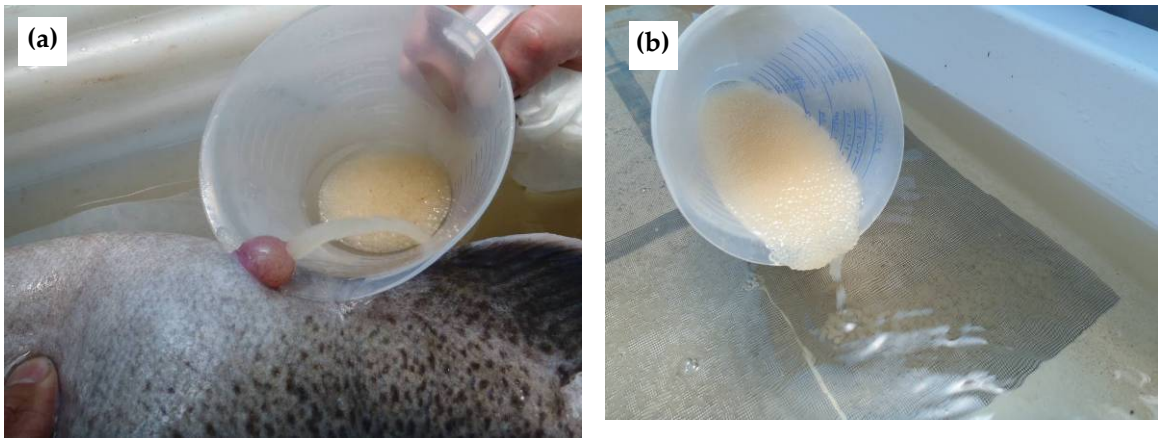

Figure 6. (a) Stripping ovulated eggs from female Murray cod. (b) Spreading fertilised eggs onto flyscreen mesh

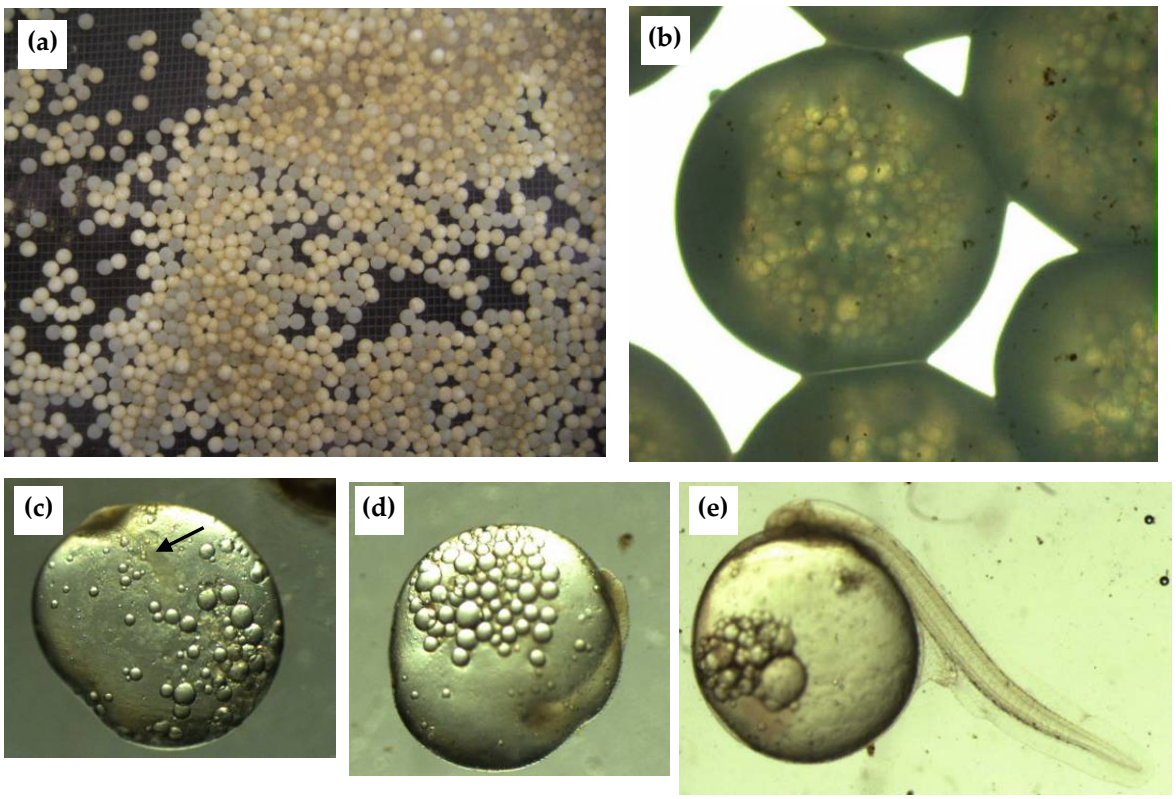

Figure 7. Developing Murray cod Murray cod eggs (a) 24 h post-fertilisation. Note viable eggs (grey eggs) and non-viable eggs (milky-white). (b) 24 h post-fertilisation. Note slightly opaque shell. (c) 44 h post-fertilisation (shell removed). Note developing embryo (arrow). (d) 69 h post-fertilisation (shell removed). (e) 122 h post-fertilisation (shell removed). Pre-hatch. Note tail free of yolk ball and developed eye orbit.

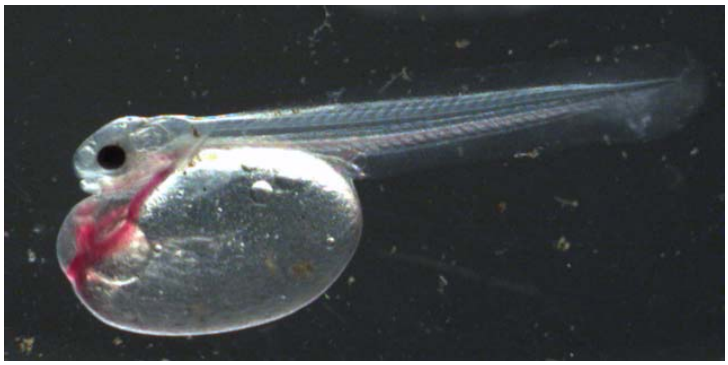

Figure 8. Hatched Murray cod larvae (Tot. lth = 8.8 mm)

Gamete quality indices, spawning evaluation parameters, and measures of spawning success

A range of indices and parameters were used to assess gamete quality and spawning success in Murray cod.

Broodstock factors

- Broodstock condition factor.
  Broodstock condition factor was determined for individual fish by applying the formula:

$$\text{Condition} = \frac{\text{Weight (g)}}{\text{total length (cm)}^3} \times 100$$

- Size (age) of broodstock at injection
- Weight of fish (kg) at time of injection
- Relative fecundity.
  Number of eggs stripped relative to body weight expressed as eggs/kg of female.
  The number of eggs stripped from each fish was determined volumetrically at the time of stripping (i.e. volume of eggs stripped), and numerically approximately 24 h after stripping by a water displacement method. Eggs, which had been adhered to strips of flyscreen mesh were lowered into specially designed tanks and the volume of water displaced recorded. After accounting for the strip of flyscreen, and determining the volume of the eggs (from measurement of egg diameter), the total number of eggs was estimated.

Gamete factors - females

- Oocyte development stage at time of hormone injection
  The proportion of oocytes that were at different stages of development, Stage S5 (Figure 3a and c), S6 (Figure 3a–d) and atretic/flaccid or resorbing (Figure 3a, e and f) at the time of hormone injection. Oocytes were sampled by canulation of ovary prior to injection
- Oocyte diameter at time of injection.
  Oocytes were sampled by canulation of ovary prior to injection. Diameter was measured with a dissecting microscope fitted with a calibrated eyepiece micrometer, or was measured from photomicrographs of samples, taken with a Allied Vision Technologies Marlin F046C digital camera fitted to a dissecting microscope, using image analysis software (ImageJ)
- Ovulated egg diameter
  Diameter of ovulated and stripped eggs at time of fertilisation
- Proportion of ovulated eggs with coalesced oil globules at fertilisation
  Oil droplet characteristics in ovulated eggs have been used previously as an oocyte quality indicator. For example, Eurasian perch with highly fragmented oil globules had lower survival rates than did eggs with one large oil globule (Żarski et al. 2011). Ovulated Murray cod eggs, were classified as coalesced when >10 oil globules were clumped together, with at >5 oil globules being medium (100–200 μm dia.) to large (>300 μm dia.) in diameter Figure 3c–e)). Small oil globules were classed as <100 μm

dia. (data collected in 2009/10 & 2011/12 only)

- Proportion of ovulated eggs with cloudy or dirty internal marks or masses (Figure 3f–g). (Data collected in 2009 & 2011 only)
  The reasons why these marks occur are not known, but may be sign a of poor quality eggs or over ripening.
  Morphological changes of over ripening in fish eggs include increased translucency, aggregation of the cortical cytoplasm along with cortical alveoli and oil droplets at the animal pole, as well as partial breakdown of cortical alveoli (Formacion et al. 1993)
- Water-hardened egg diameter
  Diameter of stripped eggs following water-hardening, 20–28 h post-fertilisation.

Gamete factors - males

- Volume of milt stripped
  The accumulated volume of milt stripped from each male during the spawning season. Note that males may be stripped more than once
- Sperm density
  The concentration of spermatozoa in stripped milts, estimated from digital imaging
- Sperm activity
  Computer assisted sperm analysis (CASA) measurements.

Measures of spawning success

- Fertilisation rate (%). Egg fertilisation rate was determined for all spawnings approximately 24 h after fertilisation. Fertilisation rate was estimated from a minimum of 4 counts of randomly selected batches of eggs. Viable, fertilised, eggs were translucent to slightly opaque and grey in colour whereas dead eggs were milky-white (Figure 7a). In some spawnings (2011/12 season only), egg viability was monitored daily from fertilisation to commencement of hatching.
- Hatch rate (%).
  The hatch rate was determined by dividing the total number of larvae by the total number of eggs for each spawning.
- Length of larvae at hatch
  The total lengths of 10-20 larvae from each spawning were measured using a dissecting microscope fitted with a calibrated eyepiece micrometer
- Larval survival rate (%)
  The percentage of hatched larvae that survive to the on-set of exogenous feeding, typically up to 2 weeks after hatch.

Other parameters

Other parameters that may have influence hatch rates that were recorded were:

- Time of spawning season
  Each spawning season was split into early mid and late
- Latency period (time between injection and stripping of gametes)
  The timing of stripping relative to ovulation is critical to the viability of the eggs. Highest viabilities are observed in eggs stripped 2-3 h after ovulation (see Rowland 1988).
- Centigrade Temperature Hours (CTH)
  The latency period (h) multiplied by the water temperature (oC) during the latency period.

Comparison of spawning success in pond-matured and tank-matured broodstock

Spawning data from tank-conditioned and artificially spawned fish (hormone induction and hand stripping of gametes) were compared with similar data gained from:

- Murray cod that spawned naturally in ponds (as described in Rowland 1983, Cadwallader and Gooley 1985). These broodstock were maintained and conditioned in earthen ponds under ambient conditions, and provided with a diet of live prey (fish and yabbies). The fish were allowed to spawn unassisted in nesting boxes located within the ponds. Historical data from 400 natural spawnings of broodstock held in earthen ponds at Snobs Creek between 1988 and 2011 were analysed
- Trout cod (*Maccullochella macquariensis*) that had been conditioned in ponds but were artificially spawned (as described Ingram and Rimmer 1992, Douglas et al. 1994). These broodstock were maintained and conditioned in earthen ponds under ambient conditions, and provided with a diet of live prey (fish and yabbies). During the spawning season, fish were captured from the ponds and artificially spawned (hormone induction and hand stripping of gametes). Historical data from 232 spawnings of broodstock between 1988 and 2006 were analysed.

Trout cod spawning data were used in this study as this species is closely related to Murray cod. These trials provided data for fish that were pond-matured then artificially spawned. Similar data for Murray cod was not readily available.

Chemical composition of eggs

In order to identify the chemical factors that affect the quality of eggs and hatch rates in Murray cod, samples of water-hardened eggs were chemically analysed. Samples of eggs were collected from:

- Pond-matured broodstock
  3 samples of water-hardened eggs from broodstock maintained in outdoor earthen ponds and fed on a natural diet (Source: commercial Murray cod hatchery).
- Tank-matured broodstock
  3 samples of water-hardened eggs from broodstock maintained in indoor tanks and fed on an artificial diet (Source: DPI Snobs Creek).

Up to 200 g of water-hardened eggs from replicate spawns, were collected approximately 24–48 h after fertilisation, drained of water then frozen at -20°C until chemical analysis was undertaken.

Chemicals analysed included mineral, vitamins, amino acids and lipids. To test for a significant difference between treatments (where $P<0.05$), analysis was undertaken using the SAS General Linear Models Procedure with Bonferroni correction (SAS Institute Inc.).

The proximate chemical composition of samples were determined via proximate composition analysis according to standard procedures, routinely implemented at Deakin University Fish Nutrition Laboratories (see Francis et al. 2007, Palmeri et al. 2007, Turchini et al. 2007). Briefly the:

- Moisture content was determined by drying samples in an oven at 80 °C to constant weight
- Protein content was determined (Kjeldahl nitrogen; N×6.25) in an automated Kjeltech (Model 2300, Tecator, Höganäs, Sweden)
- Total lipid content was determined gravimetrically after total lipid extraction by chloroform/methanol (2/1 v/v) according to Folch *et al.* (1957)
- Ash content was determined by the incineration of the sample in a muffle furnace (Model WIT, C & L Tetlow, Blackburn, Victoria, Australia) at 550 °C for 18 h.
- The fatty acid content was determined by:
  - After total lipid extraction, as described earlier, an aliquot of extracted lipids (~30mg) was used for fatty acid analysis.
  - The acid catalysed methylation method (Christie 2003) was used to esterify fatty acids into methyl esters
  - The content of fatty acid methyl esters was determined by using a Shimadzu GC 17A (Shimadzu, Chiyoda-ku, Tokyo, Japan) equipped with an Omegawax 250 capillary column (Supelco, Bellefonte, PA, USA) following a method developed and routinely used in the laboratories (see Francis et al. 2007, Palmeri et al. 2007, Turchini et al. 2007).
  - A sub-sample (~30mg) of each lipid extract was further fractionated by thin layer chromatography (TLC) to separate the four major lipid classes, namely triacylglycerol (TAG), phospholipids (PL), cholesterol esters (CE) and free fatty acids (FFA).
  - The resultant classes where then analysed for their fatty acid composition, as described above.
- Quantitative amino acid analysis including Cysteine and Tryptophan, and free amino acid analyses was undertaken by the Australian Proteome Analysis Facility (APAF) (Sydney)
  - Samples for quantitative amino acid analysis underwent 24 h liquid hydrolysis in 6M HCl at 110 °C
  - Samples for free amino acid analysis were extracted with MilliQ water and centrifuged before analysis
  - Samples for Tryptophan analysis samples underwent 24 h liquid hydrolysis in 5M NaOH at 110 °C
  - Cysteine analysis was undertaken using performic acid oxidation followed by 24 h gas phase hydrolysis at 110 °C.

    After hydrolysis all amino acids were analysed using the Waters AccQTag Ultra chemistry on a Waters ACQUITY UPLC system.

    As Asparagine is hydrolysed to Aspartic acid and Glutamine to Glutamic acid, the amount of these acids was the sum of those respective components in the Quantitative amino acid analysis.

Samples were analysed in duplicate and results are expressed as an average (Denise Thomas, APAF, pers comm.). Results were reported for quantitative amino acids and free amino acids (mg/g and mole %).

- Vitamin and mineral analysis of eggs was performed by the National Measurement Institute (Melbourne)
  - The content of the vitamins, alpha-carotene and beta-carotene, was determined using Method VL292 and Ascorbic acid by Method VL301.
  - Method VL247 was used to determine the content of the minerals magnesium, manganese, phosphorus, potassium, selenium and zinc.

Sperm evaluation and cryopreservation

Assessing sperm quality from fresh milt

In order to determine changes in sperm activity following activation with freshwater, samples of milt were collected from seven randomly selected fish. Sperm activity was assessed using CASA at 13–22 s following activation with freshwater, and then every 30 s thereafter for up to 2 min.

In order to determine changes in sperm quality following hormone injection (1,000 i.u./kg Chorulon), samples of milt were collected from five randomly selected fish at the time of injection (0 days), then at 2, 4 and 7 days after injection.

Assessing sperm quality from cryopreserved milt

After the day 7 collection of milt, each fish was stripped of milt, which was then used in cryopreservation trials.

Initial experiments indicated that Murray cod sperm could be cryopreserved in liquid nitrogen (Daly et al. 2008). However, there has been limited studies on the use of thawed sperm to fertilise eggs.

During the 2009/10 spawning season, a series of trials were conducted to further assess the effects of cryopreservation on sperm quality post-thawing, and to undertake fertilisation trials using thawed sperm.

Protocols used to freeze sperm are described in Daly et al. (2008). Briefly, milt was hand stripped from males after rinsing the abdomen and patting dry with paper towel to reduce risk of contamination of samples with water and urine. Milt was then diluted in cryoprotectant, 1 part milt with 9 parts cryoprotectant solution (90% TSK plus 10% methanol), and 100 μL of the diluent was added to each 250 μL straws (with a 100 μL air bubble at the bottom and 50 μL air bubble at the top). Up to 23 straws were used per fish. Straws were then sealed with a ball bearing. In some trials, 400 μL of diluent was added to 500 μL straws which were sealed with cotton plugs.

Straws were then plunged into -80°C for 3 minutes using a Cryologics CL 863 (Cryogenics 4 software), then transferred to pre-labelled canes and goblets which were then stored in a liquid nitrogen vessel.

Sperm was thawed by holding straws in 20°C water for 1 min before use.

Nine fertilisation trials using thawed sperm were conducted. In each trial ovulated eggs from a single female were split into two batches, one batch of eggs was fertilised with sperm freshly stripped from a male, while the second batch was fertilised with thawed sperm. Sperm that had been thawed had been stored in liquid nitrogen for 2–30 days.

To test for a significant difference between time of stripping after injection (where $P<0.05$), CASA-generated sperm activity parameters were analysed using SAS General Linear Models Procedure and Tukey's Multiple Range Test (SAS Institute Inc.). Prior to analysis, data sets that were identified as heterogeneous by using Cochran's Test for homogeneity were log transformed.

Effect of broodstock diet on spawning

Diet can have a large effect on spawning success in fish, yet there are no commercially available diets for Murray cod broodstock. Therefore, in order to evaluate the effect of broodstock diet on spawning in Murray cod, a specifically formulated broodstock diet was developed for testing.

In March 2011, approximately 9 months before the breeding season, captive-held Murray cod broodstock were randomly assigned to 10 tanks at similar densities and according to sex (6 tanks

of females and 4 tanks of males) in a RAS (Hesy) at the Snobs Creek Fish Hatchery.

Between 21 and 34 fish were stocked into each tank, giving an stocking density of approximately 95 kg/m^3.

Broodstock were fed either a commercial grow-out diet (Rid) typically fed to Murray cod, or an experimental Murray cod broodstock diet (Table 3). The latter diet was specifically formulated by Dr Giovanni Turchini (Deakin University, Warrnambool) (see Appendix II), and manufactured by the Australasian Experimental Stockfeed Extrusion Centre (AESEC) (SARDI, Adelaide).

Each diet was delivered to two tanks of males and three tanks of females, which represented approximately 50 fish of each sex per diet. Feed was provided as a % of tank biomass, which was slightly in excess of satiation, by using belt feeders or by hand.

During the breeding season (December–January) fish were spawned. Matings were conducted within treatments only (i.e. using males and females fed the same diet), using representative broodstock from each tank (chosen at random). No cross-matings between treatments were undertaken due to limited numbers of broodstock.

Gamete quality indices and spawning evaluation parameters were used to compare the effects of diets.

In addition, the specific growth rate (SGR, expressed as %/day) of individual fish was determined by using the formula:

$$\text{Specific Growth Rate (SGR)} = \frac{(\ln Wt_2 - \ln Wt_1)}{(t_2 - t_1)} \times 100\%$$

where:

t = time in days.

$\ln W_{t2}$ = natural logarithm of the average weight at time t_2.

$\ln W_{t1}$ = natural logarithm of the average weight at time t_1.

Hatched larvae from each diet treatment were stocked into replicate (3) rearing troughs and survival was monitored daily for three weeks (21 days post-hatch). Once the yolk sac had been resorbed (10–14 days post-hatch), fish were fed to excess on newly hatched brine shrimp nauplii.

Effect of hormone treatment on spawning

Hormone implants have been used extensively to enhance maturation in captive broodstock (Lee *et al.* 1986, Crim *et al.* 1988, Mylonas *et al.* 1996, Mylonas and Zohar 2001, Ingram *et al.* 2005c).

Prior to the 2011 breeding season, 30 females (15 females from each of the diet treatments described above) were implanted with Ovaplant, (Syndel Laboratories Ltd. Canada). Ovaplant is a pellet implant containing salmon Gonadotropin (sGnRHa) - Releasing Hormone analogue and it is administered to facilitate final oocyte maturation (FOM) (Figure 9).

On 2 December 2011, fish were anaesthetised and implanted with combinations of 75μg, 150 μg and 250 μg Ovaplant pellets to achieve a dose rate of 58–84 μg /kg (mean 72 μg /kg). The remaining non-implanted fish served as a control.

During the breeding season (December–January) fish were spawned. Game quality indices and spawning evaluation parameters were used to compare the effects of diets.

Analysis of spawning data matrix

In order to identify parameters and indices that affect spawning success, information from spawning trials conducted throughout the project were combined into a data matrix incorporating gamete quality indices, spawning evaluation parameters, and measures of spawning success.

Simple correlations were undertaken using Pearson product moment correlations procedure (SAS Version 9.1.3 software, SAS Institute, Inc., Cary, North Carolina, USA). These correlations were used to indicate the kind (positive or negative) and magnitude of associations between each pair of analysed parameters. Significance level was set at 0.05. Simple linear regression models were then used to describe selected significant correlations. For hatch rate, a logistic stepwise regression was used to select the most relevant and best subset of variables affecting hatch rate (level of statistical significance was 0.05).

The model is described by the resulting regression equation:

Hatch rate	=	Intercept (± s.e.)	+	Coefficient Variable 1 (± s.e.)	+	Coefficient Variable 2 (± s.e.)	+	Coefficient Variable i (± s.e.)	+	Error

Where Coefficient of Variable 1, is variable with best fit (lowest P value associated with the F statistic), Coefficient of Variable 2, is variable with next best fit (R-square), etc.

Table 3. Proximate composition of commercial diet and experimental broodstock diet used for feeding tank-reared Murray cod broodstock.

Parameter	Deakin University Broodstock diet* (DU)	Ridley Marine 45/20** (Rid)
Type	Floating	Floating
Size (mm)	20-25	15
Protein (%)	50	45
Lipid (%)	17	20
Fibre (%)		2.5
Ash (%)	5	11.2
NFE (%)	22	16
Gross energy (MJ)	23	21.29

* Detailed description is provided in Appendix II.
** Specifications from feed Manufacturer (http://www.agriproducts.com.au/)

Figure 9. Ovaplant pellets and implantation device.

Results

Broodstock

Growth

Growth of >2,800 individual broodstock (up to 8 years of age) of both sexes from 35 different families was monitored since time of tagging as juveniles.

Growth was highly variable between individuals, though regression analysis of age and weight data indicated that the growth of males was slightly higher than for females (Figure 10).

Statistical comparison of regression coefficients between sex was highly significant (P<0.0001). Regression equations for each sex were:

- Female :
  Ln(Weight) = -8.3810 + 2.2214 x Ln(Age) (Adj R2 = 0.824)
- Male:
  Ln(Weight) = -7.5879 + 2.1243 x Ln(Age) (Adj R2 = 0.779)

Where: Weight = grams and Age = days post hatch

Age at maturity

Fish were examined for maturity from mid October to mid January.

Mature, running ripe, males were first observed at 3 years of age (from 420 mm and 830 g). Mature females, that produced hatched larvae, were also first recorded at 3 years of age (from 460 mm and 1,340 g). No attempt was made to assess maturity across the entire captive population as not all fish were examined each year.

When data were compared by year class, 12% of 3 year old fish and <3% of other year classes were either immature or could not be sexed (Figure 11a).

The percent of females assessed as mature increased from 44% in 3 year old fish to 100% in 7 year old fish, then declined to 59% in 8 year old fish.

The percent of injected fish that ovulated was similar for year classes, ranging from 78–93% (Figure 11b and c). Most males (94–100%) in each year classes were running ripe (Figure 11d).

Sex ratio and hermaphrodism

A total of 1,144 fish from 33 families were sexed during the study.

Overall, there were more females (0.66) than males (0.34) (♀ : ♂ 1.91:1). In 25 families, in which >10 individuals were sexed, most families (80%) had more females than males (Figure 12).

Between 2004 and 2011, more than 650 individual fish were examined sexed and assessed for mature gametes, and of these two individuals (0.3%) from different families (tag No. 000682ED0C and 000682D8ED) were hermaphrodites. In both cases, milt containing motile spermatozoa (confirmed by microscopic examination) and mature oocytes (size 2.7-2.9 mm dia.) were observed. One female was injected with Chorulon to induce ovulation and 50 mL of eggs was stripped, but these eggs did not hatch.

Condition

Condition of broodstock examined during each spawning season ranged from 0.96–3.07 (mean 1.72 ± 0.01). A summary of condition values for broodstock of different sexes and maturity stages is provided in Table 4.

There were no significant differences in the condition of fish with different stages of maturity, and nor was condition significantly different between sexes.

Immature fish had a significantly lower condition than either mature females (with S6 oocytes) or running ripe males (P<0.0001) (Table 4).

Comparison between pond-matured and tank-matured broodstock

Historical data

The percentage of tank-matured and artificially spawned Murray cod that were induced to spawn over eight seasons ranged from 79–100% (mean 90%) (Figure 13a). This was greater than for pond-matured trout cod that were induced to spawn with hormones followed by artificial spawning (33–100%, mean 80%) and pond-matured Murray cod (mean 66%, after Ingram et al. 2011).

The fertilisation rate of tank-matured and artificially spawned Murray cod (0–100%, mean 36%) was lower than for either Murray cod that spawned naturally in ponds (1–100%, mean 89%) or pond-matured trout cod that were artificially spawned (0–100%, mean 78%) (Figure 13b).

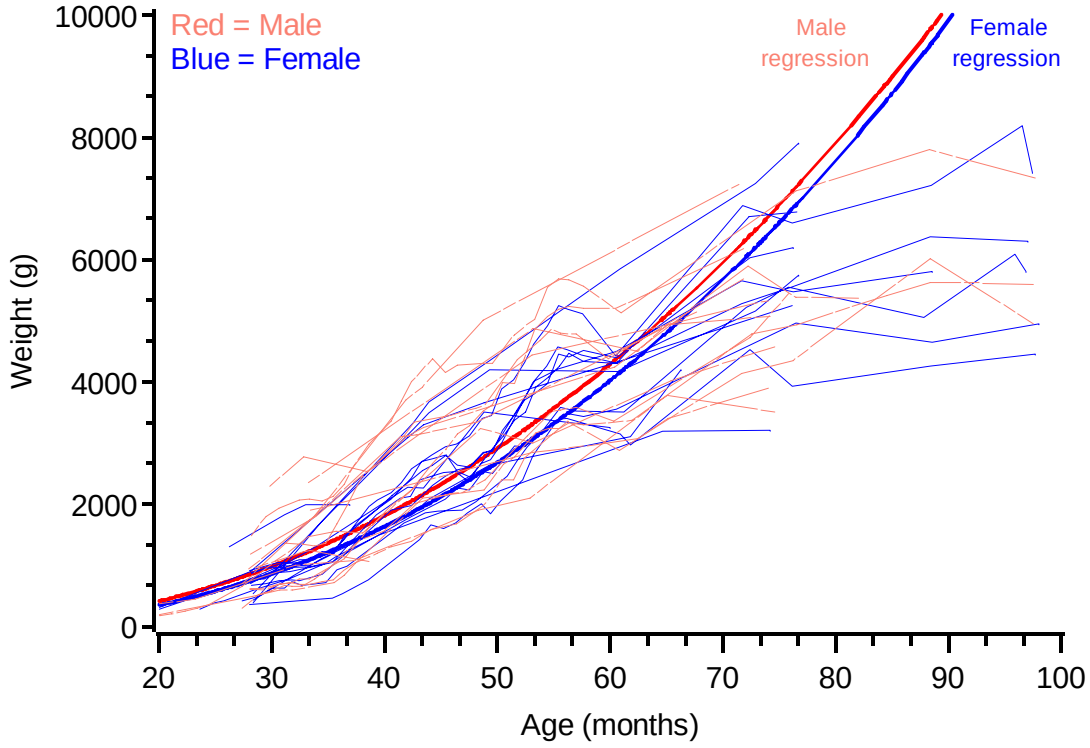

Figure 10. Growth of male and female Murray cod broodstock. Regression curves based on data for all broodstock (see text for relationships). Other data for 15 individual males and15 individual females.

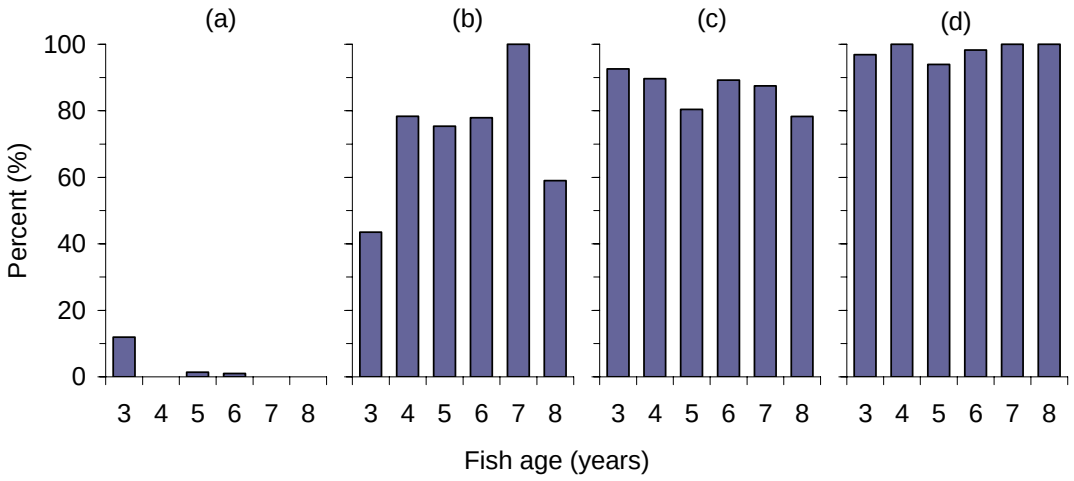

Figure 11. Maturity in Murray cod broodstock year classes. (a) Percent of fish immature or unknown sex. (b) Percent of females examined that were injected. (c) Percent of injected females that ovulated. (d) percent of males examined that were running ripe.

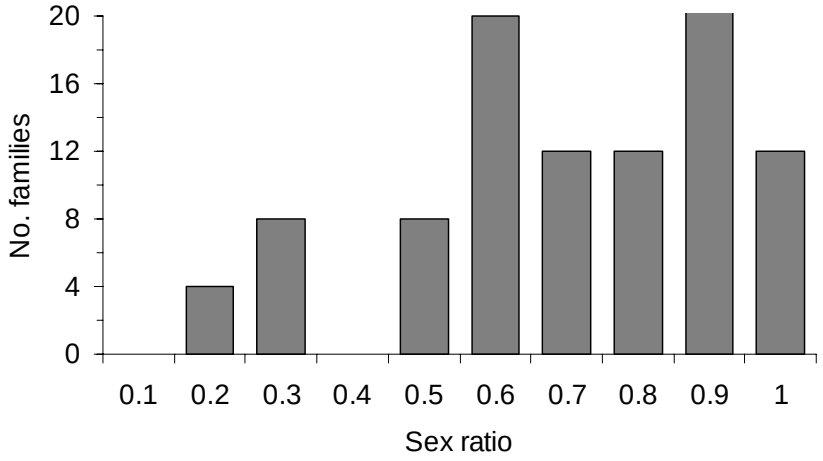

Figure 12. Sex ratio of 30 captive reared Murray cod families.

Table 4. Condition of female and male broodstock at different maturity stages.

Sex	Stage*	Condition (mean ± s.e.)
Female	Stage S5 oocytes	1.68±0.05
	Stage S6 oocytes	1.74±0.01
	Resorbing oocytes	1.74±0.02
Male	Running Ripe	1.71±0.02
	No milt	1.77±0.06
Female or male	Immature or gonad development	1.45±0.03**

* See Appendix I for definition of stages
** Value significant different to both females with S6 oocytes and running ripe males

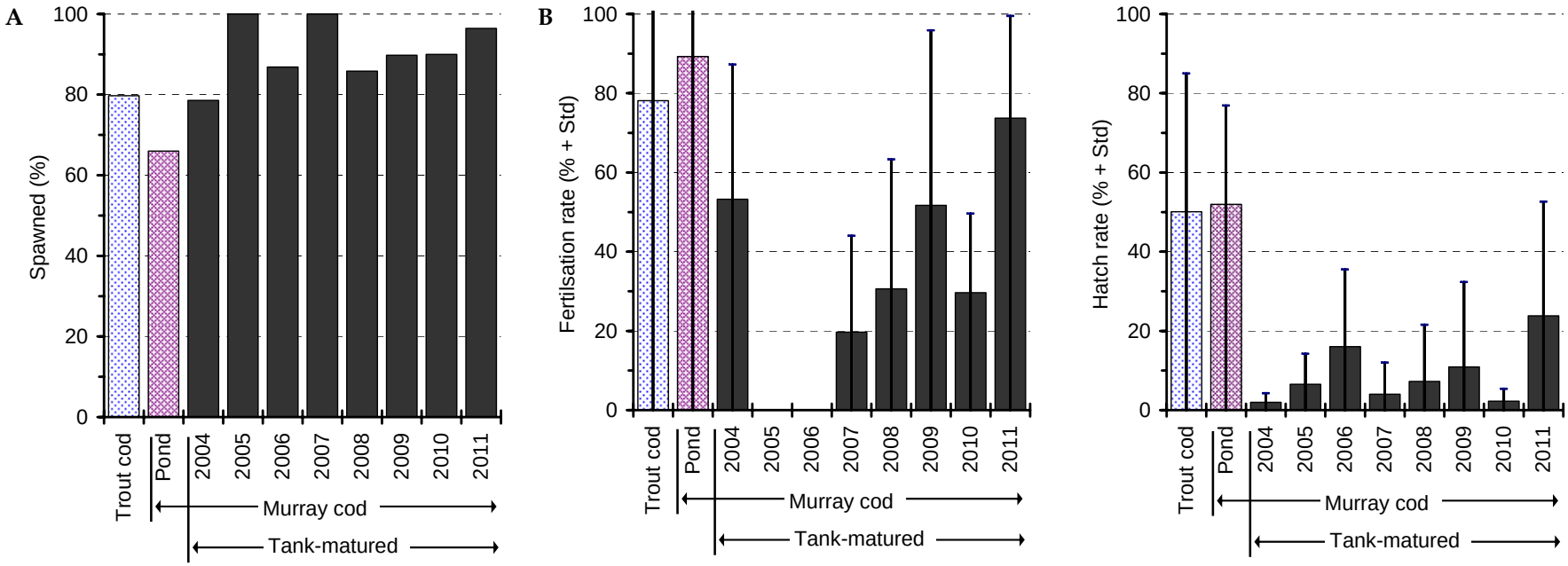

Figure 13. Comparison of percent of A) fish spawned each year, B) fertilisation rate (24 h post fertilisation) (mean ± s.e.) and C) hatch rate 9mean ± s.e.), for pond-matured and artificially spawned trout cod, pond-matured naturally spawned Murray cod ("Pond") and tank-matured and artificially spawned Murray cod.

Fertilisation rates for tank-matured fish improved over time and in the final year of spawning trials the fertilisation rate was 9–100% (mean 73%) (Figure 13b).

Hatch rates for tank-matured fish were considerably lower (0–100%, mean 9%) than for pond matured Murray cod (0–99%, mean 52%) and trout cod (0–98%, mean 50%) (Figure 13). Hatch rates did improve in tank-matured fish over the study period and in the final year of the trials were 0–96% (mean 24%).

Chemical composition of eggs

The weight of eggs produced by tank-matured fish and pond-matured fish were not significantly different (Table 5). The size of eggs produced by pond-matured fish was larger than that produced by tank-matured fish.

In general, eggs produced by tank-matured fish contained more dry matter (protein and lipid), and less moisture than did eggs produced by pond-matured fish (Table 5). Significant differences were found in:

- Moisture content
- Lipid, protein and dry matter (as mg/g on wet weight)
- Ash content (as % dry weight).

Mineral content

The Magnesium, Phosphorus, Selenium, and Zinc content of eggs produced by pond-matured fish was significantly lower ($P<0.05$) than that for eggs produced by tank-matured fish, while Manganese was significantly higher. There was no significant difference in the content of Potassium in eggs produced by tank- and pond-matured (Table 5).

Vitamin content

The content of alpha-Carotene, Ascorbic acid and beta-Carotene in eggs of fish reared in tanks and in ponds were below detectable levels (Table 5).

Fatty acid content

The overall total fatty acid composition (mg of fatty acid per g of lipid) of egg is reported in Table 6.

Major differences were noted between the two samples of eggs, with several statistically significant differences recorded.

The most salient findings were that eggs produced from pond-matured fish had a significantly higher content of 18:1n-9, 20:4n-6, 22:4n-6, MUFA, n-6 PUFA, and n-6 LC-PUFA than did eggs produced from tank-matured fish.

Eggs produced from tank-matured fish had higher content of 20:5n-3, 22:5n-3, 22:6n-3, PUFA and total n-3 LC-PUFA.

Fatty acid composition

The fatty acid composition (% of total FA w/w) for different lipid classes (phospholipids, triacylglycerol, cholesterol esters and free fatty acids) of Murray cod eggs from different sources is reported in Table 7.

Major differences were noted between the two samples of eggs, with several statistically significant differences recorded.

Amino acid composition

Differences in amino acid composition of eggs from pond-matured fish and tank-matured fish are presented in Table 8.

There were significant differences in the content of most of the free amino acids and all of the quantitative amino acids in eggs derived from pond-matured and tank matured fish.

The content of total quantitative amino acids was significantly higher in eggs produced by tank-matured fish than in eggs produced by pond-matured fish (Tank-matured: mean = 230 mg/g. Pond matured: mean = 161 mg/g).

The content of total free amino acids was not significantly different between eggs derived from pond- and tank matured fish (Table 8).

Oocyte and egg evaluation

Oocyte samples collected at the time of initial inspection (pre-injection) contained mainly S6 oocytes with varying proportion of S5 and resorbing oocytes. Stage S5 oocytes were typically smaller than S6 oocytes whereas resorbing oocytes were larger, more variable in size, flaccid and often clear (Table 9, Figure 3e and f).

The time between hormone injection and fertilisation of eggs following ovulation and stripping was 45–57.8 h (mean 49.6) (water temperature 19–22.5°C). Centigrade Temperature Hours (CTH) ranged from 923–1,135 (Table 9).

Ovulated oocytes were 2.42–3.56 mm in dia. (mean 3.03 mm), and the proportion of these with coalesced oil globules was highly variable (0.07–1) while few had cloudy or dirty marks visible internally (0–0.64) (Table 9). The fertilisation rate of stripped eggs, assessed 24 h after fertilisation, was 0–99.6 % (mean 36 %). At this stage eggs that had water-hardened were 2.35–3.92 mm dia. (mean 3.19 mm dia.).

Table 5. Proximate, mineral and vitamin analysis of eggs from pond-matured naturally spawned Murray cod and tank-matured and artificially spawned Murray cod. Data reported as mean ± s.e. (Sign= significance – Bonferroni correction).

Parameter	Tank-matured	Pond-matured	Sign.
Egg weight (mg)	14.1 ± 0.55	15.88 ± 1.25	0.2639
Moisture (mg/g wet wt)	**682 ± 3**	**763 ± 10**	**0.0014**
Ash (mg/g wet wt)	5.77 ± 0.74	7.51 ± 0.47	0.1177
Lipid (mg/g wet wt)	**51.43 ± 3.12**	**35.42 ± 1**	**0.0081**
Protein (mg/g wet wt)	**261 ± 6**	**194 ± 9**	**0.0039**
Dry matter (mg/g wet wt)	**318 ± 3**	**237 ± 10**	**0.0014**
Ash (% dry wt)	**1.82 ± 0.25**	**3.17 ± 0.25**	**0.0178**
Lipid (% dry wt)	16.17 ± 1.09	14.96 ± 0.49	0.3652
Protein (% dry wt)	82.01 ± 1.3	81.87 ± 0.74	0.9307
Protein - lipid ratio	5.12 ± 0.4	5.49 ± 0.22	0.4723
Magnesium (mg/kg)	**180 ± 10**	**113.33 ± 8.82**	**0.0075**
Manganese (mg/kg)	**0.21 ± 0.06**	**3.77 ± 0.82**	**0.0123**
Phosphorus (mg/kg)	**2767 ± 167**	**1500 ± 100**	**0.0029**
Potassium (mg/kg)	164 ± 120	593 ± 109	0.057
Selenium (mg/kg)	**0.5 ± 0.01**	**0.31 ± 0.03**	**0.0035**
Zinc (mg/kg)	**20.67 ± 2.19**	**9.5 ± 0.4**	**0.0074**
Alpha-Carotene (µg/100g)	<5	<5	
Ascorbic acid (µg/100g)	<1.0	<1.0	
Beta-Carotene (µg/100g)	<5	<5	

Legend:

Cells shaded green indicate significantly higher concentrations were detected in eggs produced from pond- matured fish

Cells shaded orange indicates significantly higher concentrations were detected in eggs produced from tank-matured fish

Table 6. Total fatty acid composition (mg of FA/ g of lipid) of eggs from pond-matured naturally spawned Murray cod and tank-matured and artificially spawned Murray cod. Data reported as mean ± s.e. (Sign= significance – Bonferroni correction).

Fatty acid	Tank-matured	Pond-matured	Sign.
14:0	7.92 ± 0.71	4.8 ± 0.32	0.016
16:0	91.51 ± 4.06	88.47 ± 1.26	0.5146
16:1n-7	25.41 ± 1.54	17.83 ± 1.39	0.0218
18:0	38.48 ± 0.11	49.7 ± 2.5	0.011
18:1n-9	99.3 ± 3.09	132 ± 0.66	0.0005
18:1n-7	22.69 ± 0.79	20.83 ± 0.76	0.1631
18:2n-6	26.67 ± 2.1	32.08 ± 0.36	0.0643
18:3n-6	1.7 ± 0.33	2.81 ± 0.19	0.042
18:3n-3	2.93 ± 0.13	4.75 ± 2.03	0.4205
18:4n-3	2.16 ± 0.16	0.91 ± 0.11	0.0031
20:0	1.17 ± 0.03	0.9 ± 0	0.0013
20:1n-11	1.18 ± 0.48	1.29 ± 0.11	0.8411
20:1n-9	2.58 ± 0.28	3.51 ± 0.3	0.0878
20:2n-6	1.28 ± 0.07	2.03 ± 0.07	0.0019
20:3n-6	0.96 ± 0.02	4.01 ± 0.69	0.0116
20:4n-6	9.76 ± 0.37	23.08 ± 4.18	0.0336
20:3n-3	0.57 ± 0.03	1.17 ± 0.25	0.0725
20:4n-3	3.36 ± 0.13	2.1 ± 0.19	0.0054
20:5n-3	34.89 ± 2.05	11.57 ± 1.14	0.0006
22:1n-11	0.19 ± 0.01	0 ± 0	0.0002
22:1n-9	0.29 ± 0.05	0.32 ± 0.2	0.9153
22:4n-6	1.76 ± 0.09	4.89 ± 0.96	0.0311
22:3n-3	3.05 ± 0.01	3.11 ± 0.29	0.8568
22:5n-3	24.86 ± 1.18	15.87 ± 0.82	0.0033
22:6n-3	105 ± 4	68 ± 5	0.0053
Total	509 ± 11	496 ± 7	0.364
SFA	139 ± 3	144 ± 4	0.4001
MUFA	152 ± 1	176 ± 2	0.0004
PUFA	219 ± 7	176 ± 3	0.0062
n-6 PUFA	42.14 ± 2.8	68.92 ± 5.88	0.0147
n-6 LC-PUFA	13.77 ± 0.52	34.02 ± 5.76	0.0248
n-3 PUFA	177 ± 5	107 ± 4	0.0005
n-3 LC-PUFA	171 ± 5	102 ± 6	0.0009
n-3/n-6 PUFA	4.21 ± 0.19	1.59 ± 0.2	0.0007
n-3/n-6 LC-PUFA	12.47 ± 0.27	3.22 ± 0.69	0.0002

Legend:

Cells shaded green indicate significantly higher concentrations were detected in eggs produced from pond-matured fish

Cells shaded orange indicate significantly higher concentrations were detected in eggs produced from tank-matured fish

Table 7. Fatty acid lipid class composition (% of total FA w/w) of eggs from pond-matured naturally spawned Murray cod and tank-matured and artificially spawned Murray cod. Data reported as mean ± s.e.E. (Sign= significance – Bonferroni correction) (nd= no data).

Fatty acid	Phospholipids (PL)			Triacylglycerol (TAG)			cholesterol esters (CE)			Free fatty acids (FFA)		
	Tank-matured	Pond-matured	Sign.	Tank-matured	Pond-matured	Sign.	Tank-matured	Pond-matured	Sign.	Tank-matured	Pond-matured	Sign.
14:0	1.19 ± 0.04	0.91 ± 0.11	0.0656	3.05 ± 0.62	2.38 ± 0.45	0.4279	0.49 ± 0.12	0.73 ± 0.05	0.1413	1.94 ± 0.33	1.5 ± 0.41	0.4496
14:1n-5	0.03 ± 0.01	0.08 ± 0.02	0.0671	0.05 ± 0.03	0.08 ± 0	0.3965	0.14 ± 0.14	0.22 ± 0.12	0.6759	0.18 ± 0.09	0.14 ± 0.14	0.827
16:0	21.85 ± 1.12	19.18 ± 1.34	0.2024	23.68 ± 1.02	25.15 ± 1.02	0.3669	3.95 ± 0.65	6.88 ± 1.2	0.098	17.73 ± 1.21	23.85 ± 2.38	0.0839
16:1n-7	2.26 ± 0.05	2.29 ± 0.26	0.9179	9.83 ± 1.35	7.94 ± 0.68	0.2816	9.12 ± 1.65	6.12 ± 0.19	0.1444	**5.41 ± 0.72**	**2.92 ± 0.32**	**0.034**
18:0	10.21 ± 0.13	12.92 ± 1.32	0.1111	4.75 ± 0.21	5.06 ± 0.42	0.5425	2.64 ± 0.35	6.56 ± 2.27	0.1632	8.14 ± 1.76	13.49 ± 2.19	0.1294
18:1n-9	**14.67 ± 0.56**	**20.72 ± 0.9**	**0.0047**	**27.57 ± 0.49**	**33.53 ± 1.99**	**0.0436**	29.01 ± 2.29	33.98 ± 2.22	0.1938	13.47 ± 0.79	15.11 ± 2.07	0.4981
18:1n-7	**4.56 ± 0.21**	**3.62 ± 0.18**	**0.0288**	5.07 ± 0.23	4.68 ± 0.08	0.1776	3.93 ± 0.26	2.92 ± 0.68	0.2387	3.01 ± 0.12	3.43 ± 0.3	0.2571
18:2n-6	**2.69 ± 0.36**	**6.39 ± 0.2**	**0.0009**	7.29 ± 0.29	7.89 ± 0.19	0.1657	14.28 ± 0.84	12.89 ± 0.85	0.3089	5.21 ± 0.42	4.09 ± 1.01	0.3668
18:3n-6	**0.27 ± 0.01**	**0.56 ± 0.03**	**0.0008**	**0.42 ± 0.02**	**0.7 ± 0.09**	**0.0395**	0.62 ± 0.04	0.57 ± 0.03	0.4748	nd	0.13 ± 0.07	
18:3n-3	0.24 ± 0.02	0.7 ± 0.19	0.0721	0.68 ± 0	1.27 ± 0.61	0.3932	2 ± 0.03	3.31 ± 1.18	0.3274	1.14 ± 0.16	0.76 ± 0.05	0.0814
18:4n-3	0.2 ± 0.01	0.72 ± 0.27	0.1241	0.54 ± 0.08	0.5 ± 0.2	0.8525	1.28 ± 0.12	1.66 ± 0.23	0.2142	0.47 ± 0.26	0.74 ± 0.24	0.4911
20:0	0.16 ± 0.01	0.19 ± 0.04	0.5964	0.22 ± 0.02	0.18 ± 0.02	0.2378	0.23 ± 0.1	0.6 ± 0.12	0.0779	0.22 ± 0.11	0.21 ± 0.21	0.9684
20:1n-11	nd	0.34 ± 0.08		0.07 ± 0.04	0.28 ± 0.09	0.1008	0.58 ± 0.38	2.13 ± 0.56	0.0835	nd	nd	
20:1n-9	0.58 ± 0.04	0.53 ± 0.02	0.3564	0.69 ± 0.1	0.69 ± 0.11	0.9788	0.97 ± 0.16	0.75 ± 0.29	0.557	3.3 ± 0.39	2.24 ± 0.77	0.285
20:2n-6	0.7 ± 0.05	1.45 ± 0.45	0.1779	0.6 ± 0.05	1.4 ± 0.56	0.2229	2.19 ± 0.23	4.16 ± 0.74	0.0656	8.22 ± 0.94	4.85 ± 0.82	0.054
20:3n-6	**0.18 ± 0.01**	**0.99 ± 0.16**	**0.0072**	**0.18 ± 0.02**	**0.43 ± 0.04**	**0.0038**	0.31 ± 0.06	0.79 ± 0.16	0.0527	**0.25 ± 0.12**	**0.95 ± 0.01**	**0.0049**
20:4n-6	**2.55 ± 0.05**	**6.75 ± 1**	**0.0139**	**1.08 ± 0.09**	**1.71 ± 0.19**	**0.0412**	1.75 ± 0.25	2.99 ± 0.42	0.0668	2.69 ± 0.17	2.5 ± 0.7	0.8068
20:3n-3	0.15 ± 0.02	0.22 ± 0.09	0.4743	0.14 ± 0.04	0.17 ± 0.02	0.4832	0.39 ± 0.05	0.69 ± 0.28	0.3504	1.68 ± 0.2	1.4 ±0.22	0.3962
20:4n-3	0.28 ± 0.01	0.31 ± 0.03	0.4514	**0.43 ± 0.02**	**0.24 ± 0.03**	**0.0029**	1.04 ± 0.1	0.67 ± 0.28	0.2802	0.26 ± 0.26	1.03 ± 0.43	0.1985
20:5n-3	**9.72 ± 0.77**	**3.43 ± 0.45**	**0.0021**	**2.48 ± 0.13**	**0.78 ± 0.11**	**0.0006**	**9.88 ± 1.65**	**2.91 ± 0.5**	**0.0155**	**5.32 ± 0.55**	**1.45 ± 0.62**	**0.0095**
22:0	0.06 ± 0.01	nd		nd	nd		nd	nd		nd	0.25 ± 0.25	
22:1n-11	0.11 ± 0.01	0.18 ± 0.04	0.1661	0.08 ± 0.02	0.03 ± 0.02	0.0928	0.23 ± 0.13	nd		nd	0.33 ± 0.22	
22:1n-9	**0.12 ± 0**	**0.19 ± 0.02**	**0.0253**	0.04 ± 0.03	0.06 ± 0.05	0.7633	0.44 ± 0.24	1.42 ± 1.42	0.533	**6.46 ± 1.07**	**2.57 ± 0.43**	**0.028**
22:2n-6	0.02 ± 0.01	nd		0.01 ± 0.01	nd		nd	nd		0.09 ± 0.09	0.08 ± 0.08	0.9032
22:4n-6	**0.23 ± 0.01**	**0.72 ± 0.18**	**0.0491**	0.08 ± 0.01	0.16 ± 0.04	0.1629	0.09 ± 0.06	nd		nd	0.23 ± 0.23	
22:3n-3	0.13 ± 0.02	0.43 ± 0.19	0.199	0.21 ± 0.05	0.25 ± 0.07	0.7287	0.6 ± 0.2	1.36 ± 0.31	0.1098	4.47 ± 0.85	5.88 ± 3.35	0.7047
22:5n-3	**4.03 ± 0.07**	**2.41 ± 0.25**	**0.0031**	2.39 ± 0.53	1.13 ± 0.15	0.0819	3.75 ± 0.97	1.07 ± 0.21	0.0536	2.27 ± 0.21	2.26 ± 0.72	0.996
22:6n-3	**22.82 ± 0.92**	**13.76 ± 0.99**	**0.0026**	8.34 ± 1.66	3.33 ± 1.76	0.1069	10.09 ± 2.7	4.64 ± 1.32	0.144	8.08 ± 0.35	7.25 ± 2.67	0.7741

Cells shaded green indicate significantly higher concentrations were detected in eggs produced from pond-matured fish

Cells shaded orange indicates significantly higher concentrations were detected in eggs produced from tank-matured fish

Table 8. Free and quantitative amino acid composition of eggs from pond-matured naturally spawned Murray cod and tank-matured and artificially spawned Murray cod. Data reported as mean ± s.e. (Sign=significance – Bonferroni correction).

Amino acid	Free Amino acids* (mg/g)			Quantitative Amino acids** (mg/g)		
	Tank-matured	Pond-matured	Sign.	Tank-matured	Pond-matured	Sign.
Histidine	44 ± 6.93	24 ± 2.31	0.052	8 ± 0.1	5.8 ± 0.2	0.0006
Asparagine	5.67 ± 1.2	1.33 ± 0.33	0.0255			
Serine	3.33 ± 1.33	131 ± 2	<0.0001	13 ± 0.06	8.67 ± 0.42	0.0005
Glutamine	2.67 ± 0.88	8.67 ± 3.18	0.1432			
Arginine	8 ± 2.31	29.33 ± 9.13	0.0863	15.67 ± 0.13	10.57 ± 0.57	0.0009
Glycine	46 ± 8	53.33 ± 7.45	0.539	5.47 ± 0.03	3.7 ± 0.2	0.001
Aspartic acid	6 ± 1.53	82 ± 4.51	<0.0001	17.8 ± 0.21	12.43 ± 0.64	0.0014
Glutamic acid	112 ± 3	129 ± 5	0.0337	27.43 ± 0.27	19.9 ± 0.85	0.0011
Threonine	11.33 ± 2.85	47.33 ± 5.81	0.0051	11.63 ± 0.07	8 ± 0.4	0.0009
Alanine	215 ± 7	162 ± 4	0.0024	16.93 ± 0.12	11.7 ± 0.7	0.0018
Proline	72.67 ± 2.33	55.33 ± 1.76	0.0041	12.6 ± 0.21	9.8 ± 0.21	0.0007
Cysteine	8 ± 1.53	9.67 ± 1.67	0.5019	2.7 ± 0.32	1.67 ± 0.09	0.0362
Lysine	153 ± 7	120 ± 6	0.026	19.67 ± 0.19	13.37 ± 0.72	0.0011
Tyrosine	20.33 ± 3.93	7 ± 1	0.0303	9.27 ± 0.07	6.6 ± 0.25	0.0005
Methionine	90.67 ± 3.76	57 ± 2.31	0.0016	6.63 ± 0.03	4.53 ± 0.23	0.0009
Valine	144 ± 5	117 ± 2	0.0099	15.3 ± 0.12	10.77 ± 0.47	0.0007
Isoleucine	151 ± 9	103 ± 1	0.0057	14.07 ± 0.13	9.67 ± 0.57	0.0016
Leucine	310 ± 16	185 ± 6	0.002	22.97 ± 0.19	16 ± 0.85	0.0013
Phenylalanine	117 ± 5	71 ± 4	0.0017	8.5 ± 0.06	5.87 ± 0.27	0.0006
Tryptophan	21.33 ± 0.67	24 ± 2.08	0.2895	2.53 ± 0.03	1.7 ± 0.06	0.0002
TOTAL AA	1543 ± 43	1417 ± 30	0.0744	230 ± 2	161 ± 8	0.0009

* = Calculation based on free amino acid molecular weight.
** = Calculation based on amino acid residue mass in protein (molecular weight minus H_2O)
Cells shaded green indicates significantly higher concentrations were detected in eggs produced from pond-matured fish
Cells shaded orange indicate significantly higher concentrations were detected in eggs produced from tank-matured fish

Table 9. Summary of Murray cod oocyte, egg and hatched larvae measurements.

Parameter		Range	Mean ± s.e.
Oocytes pre-injection	S6 diameter (mm)	2.22–3.3	2.86 ± 0.009
	S6 proportion of sample	0–1	0.51 ± 0.02
	S5 diameter (mm)	1.27–2.26	1.72 ± 0.05
	S5 proportion of sample	0–1	0.2 ± 0.01
	Resorbing oocyte diameter (mm)	2.78–4.06	3.49 ± 0.1
	Resorbing proportion of sample	0–1	0.31 ± 0.02
Ovulated oocytes	Diameter (mm)	2.42–3.56	3.03 ± 0.01
	Proportion coalesced	0.07–1	0.51 ± 0.02
	Proportion with cloudy/dirt mark	0–0.64	0.14 ± 0.02
Stripped eggs	Volume (mL)	3–425	141 ± 5
	Number	150–26,576	8,790 ± 302
	Relative fecundity (eggs/kg)	19–4,920	1,986 ± 53
Water-hardened eggs (24hrs post-fertilisation)	Diameter (mm)	2.35–3.92	3.19 ± 0.01
	Weight (mg)	10–24.5	15.4 ± 0.3
Fertilisation rate (%)		0–99.6	36.4 ± 2.3
Hatch rate (%)		0–99.97	8.68 ± 1.15
Hatched larvae (mmTL)		6.4–10.5	8.78 ± 0.04
Latency (h)		45–57.8	49.6 ± 0.1
Centigrade Temperature Hours (CTH)		923–1,135	1,025 ± 4

There was a slight increase in egg diameter from pre-injection (S6 stage) and ovulated to 24 h poste fertilisation (Figure 14a). In the days following fertilisation, the percent of eggs that remained viable (i.e. had not died) declined at various rates. Most mortalities occurred in the first three days following fertilisation (Figure 14b).

Hatch rates were highly variable 0–99.97%, but on average low (9%). At hatch, larvae were 6.4–10.5 mm (mean 8.8 mm) in length (Figure 8).

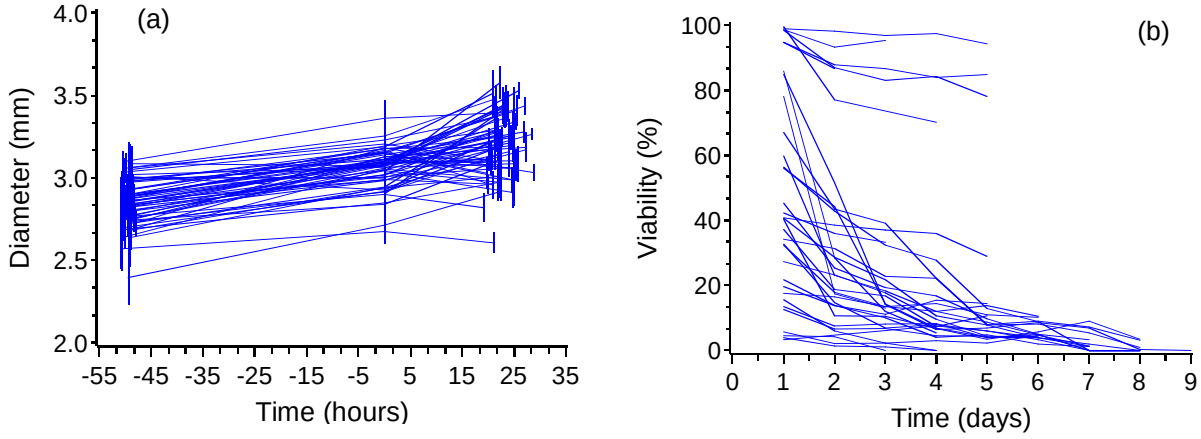

Figure 14. (a) Change in egg size following fertilisation and water-hardening (Values represent mean ± s.e. for each batch of eggs). (b) Change in viability of eggs during incubation.

Sperm evaluation and cryopreservation

Sperm morphology and characteristics

The results of the qualitative and quantitative assessment of sperm are provided in Table 10.

Sperm density in milt varied considerably ranging from from $3.7x10^6$ cells/μL to $462x10^6$ cells/μL (mean $61x10^6$ cell/ μL). Based on these values the amount of sperm used to fertilise eggs ranged from 2.5 – 172.5 x 10^6 sperm/egg (mean 26.9 x 10^6 sperm/egg).

There was a strong positive relationship between the subjective sperm consistency measure and objective cell density measure (Figure 15). From 107 samples, sperm motility ranged from 0 % to 100 % (mean 59 %) (Table 10).

The volume of milt used to fertilise Murray cod eggs was influenced by the available males at the time if spawning, and the amount of milt that could be stripped from those fish. In most cases (90%) a single male was used, but on some occasions (where single crosses were not required) milt from up to two males were used to fertilise eggs. Some males were stripped up to three times during the season. The amount of milt stripped from males and used to fertilise eggs ranged from 0.1–5.2 mL (mean 1.47 ± 0.1 s.e.) (Table 10). The relationship between volume of milt stripped and fish weight was:

Volume stripped (mL) = 0.486 + 0.002 x Fish weight (g) (Adj. R2= 0.086, P=0.0007) (Figure 16).

There was no clear seasonal pattern in sperm density (Figure 17), but sperm motility increased towards the middle of the spawning season then declined towards the end in both years of the study (Figure 17).

The motility of sperm declined dramatically following activation in freshwater. When averages across all nine fish were examined, motility at 103–111 seconds after activation was just 5% of that recorded within the first 30 seconds following activation (Figure 18).

Examination of spermatozoa using phase contrast microscopy showed sperm cells in which the flagella appeared to be looped at the end. These abnormal cells were detected in the milt of most of the fish examined (Figure 19). This abnormal morphology has not been recorded previously.

The volume of milt that could be stripped from fish increased in the days immediately following injection with hormone. Sperm density in stripped milt changed significantly (P=0.0188) in the days following injection (Table 11). Sperm density was lowest 2 days following injection and then increased.

Motility of sperm in the days following injection was not significantly different to that at the time of injection, and was maintained for at least 7 days following injection (Table 11, Figure 20). Similarly, there was minimal variation (non-significant) between CASA sperm measures for sperm analysed before injection and up to 7 days after injection (Table 11, Figure 21). However, there was a noticeable decline in both milt volume and between 7 and 14 days post injection. Sperm consistency generally did not change over time from pre-injection to 14 days post injection.

Sperm cryopreservation trials 2010/11

There is a significance difference (P=0.0054) in the percentage of sperm cells motility between fresh sperm and thawed sperm that what collected at different times post hormone injection of broodstock (Figure 20).

The motility of the fresh sperm, collected before the administration of the hormone injection was 67 %. This sample was then frozen in liquid nitrogen. Upon thawing, the motility in the same samples was 3 % (Table 12).

Analysis of sperm motility indices using CASA showed that there were no observable differences between fresh and frozen sperm for other indices (Table 12, Figure 21). Although not significant VSL was consistent across all treatments for fresh sperm, but was more variable and on average slightly higher for thawed sperm (Figure 21).

Use of large straws for freezing milt was not successful, as on thawing the seals failed and frozen sperm was ejected from the straws before it could be used.

Table 10. Assessment measures and characteristics of Murray cod Sperm recorded during the current project (see general methods for abbreviation definitions).

Parameter	Mean ± s.e.	Range	No. observations
Sperm density (cells/μL x 10^6)	61.22 ± 6.02	3.7–462.12	102
Volume of milt used to fertilise eggs (mL)	1.47 ± 0.1	0.1–5.2	150
Sperm to egg (sperm x 10^6/egg)	29.6 ± 8.5	2.5 – 172.5	20
Subjective measures			
Milt Volume	1.91 ± 0.09	0–3	102
Sperm Consistency	2.46 ± 0.06	1–4	94
Sperm Activity	2.4 ± 0.11	0–4	65
CASA measures			
Motility (%)	58.96 ± 3.37	0–100	107
VCL	102.6 ± 4.96	35.50–191.2	92
VAP	49.29 ± 1.26	25.9–77.0	92
VSL	28.93 ± 2.52	5.0–122.1	92
LIN	0.54 ± 0.04	0.12–2.31	92
WOB	0.55 ± 0.02	0.34–0.98	92
PROG	158.6 ± 7.31	40.7–373.0	92
BCF	8.57 ± 0.32	2.91–19.3	92

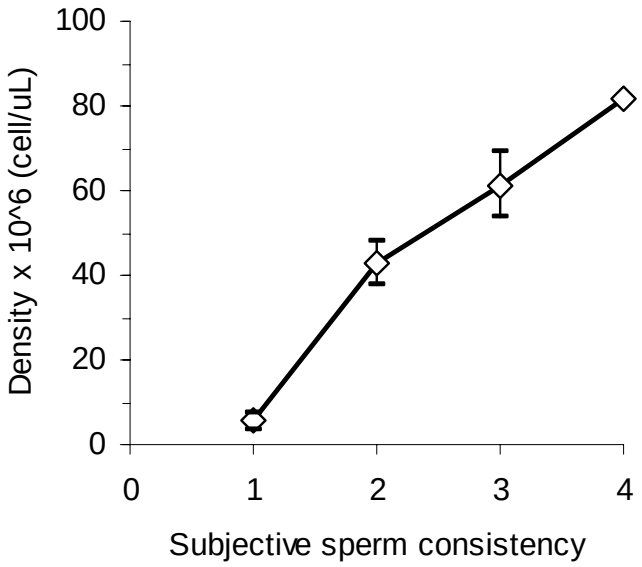

Figure 15. Relationship between subjective sperm consistency measure (see Table 2) and objective cell density measure.

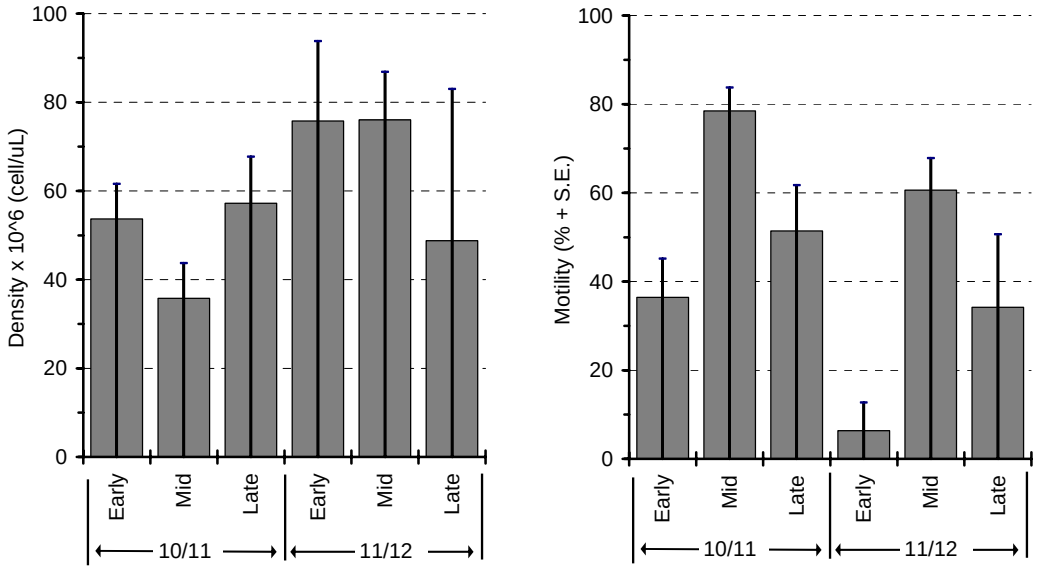

Figure 16. Seasonal variation in sperm density and sperm motility of Murray cod (values = mean ± s.e.).

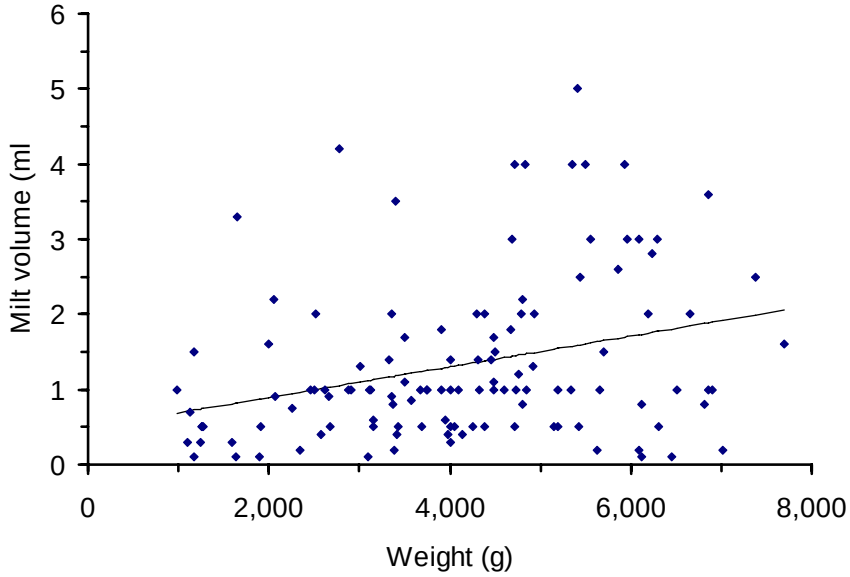

Figure 17. Relationship between fish weight and volume of milt stripped (Volume stripped (mL) = 0.486 + 0.002 • Fish weight (g) (Adj. R^2= 0.086, P=0.0007).

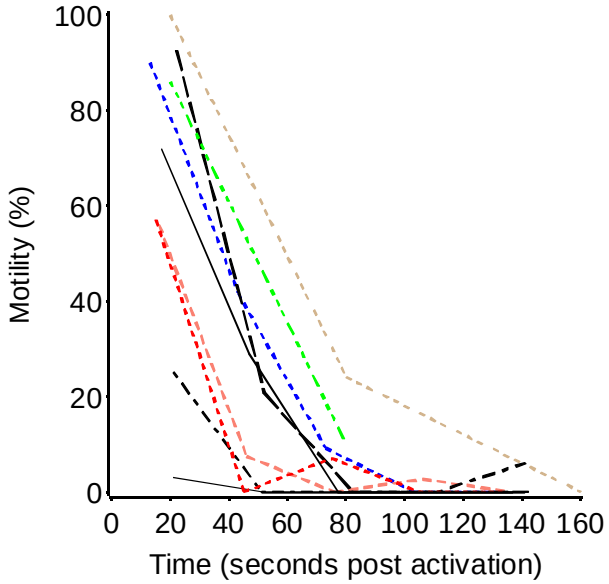

Figure 18. Change in motility of Murray cod sperm following activation in freshwater.

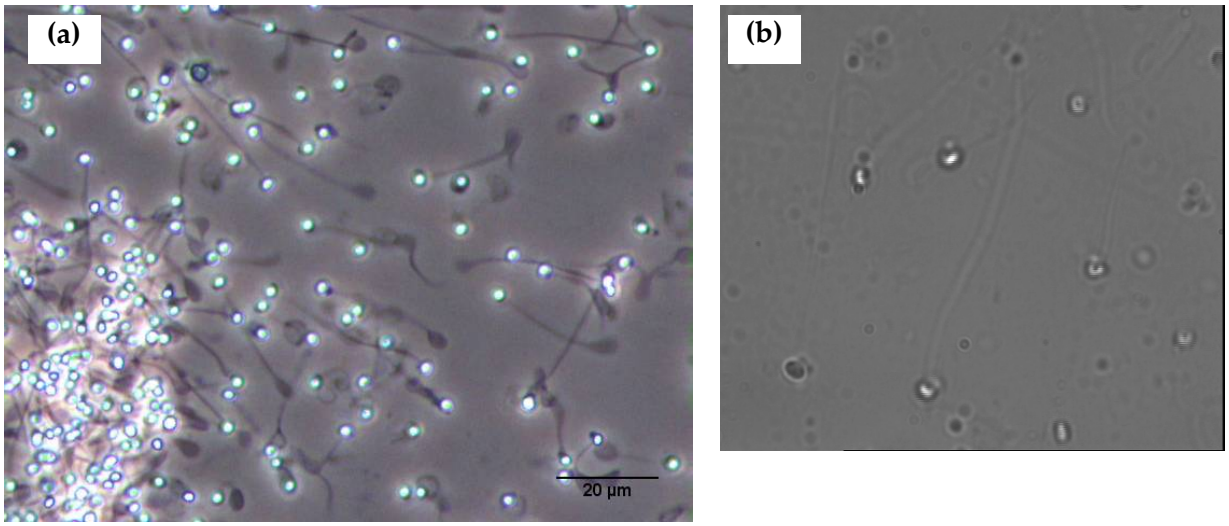

Figure 19. Murray cod spermatozoa (Phase contrast). (a) Sperm cells with looped flagella. (b) sperm cells with straight flagella.

Table 11. Changes in sperm characteristic over time following injection of hormone to enhance spermiation in Murray cod (Values = mean ± S.E).

Parameter	Pre-injection	Post-injection			Sign
		2 days	4 days	7 days	
Density (cells/µL x 10^6)	62.79 ± 10.62[a]	20.45 ± 3.27[b]	59.16 ± 36.98[ab]	58.5 ± 12.62[ab]	0.0188
Motility (%)	54.42 ± 8.98	54.72 ± 10.15	73.74 ± 7.39	45.83 ± 14.05	0.7169
VCL	75.49 ± 12.4	59 ± 11.8	56.89 ± 2.74	76.24 ± 16.91	0.7652
VAP	43.16 ± 3.3	37.63 ± 2.96	41.43 ± 2.25	42.33 ± 3.78	0.6966
VSL	14.82 ± 2.87	12.46 ± 3.15	9.06 ± 1.44	22.85 ± 11.78	0.5735
LIN	0.32 ± 0.04	0.32 ± 0.06	0.22 ± 0.05	0.48 ± 0.21	0.5204
WOB	0.65 ± 0.04	0.72 ± 0.06	0.73 ± 0.07	0.63 ± 0.07	0.6821
PROG	109.24 ± 15.02	102.31 ± 14.99	84.11 ± 3.72	139.1 ± 28.49	0.5299
BCF	6.61 ± 0.63	5.16 ± 0.49	5.92 ± 0.31	7.17 ± 1.01	0.3204

Cells shaded in grey in significant differences in parameters between pre and post injection

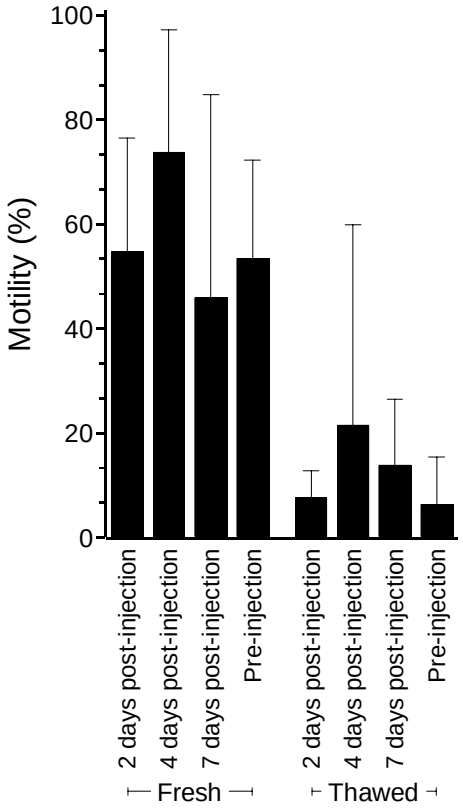

Figure 20. Motility (%) of fresh sperm before hormone injection, and 2, 4 and 7 days after hormone injection, and thawed sperm that was frozen before hormone injection, and 2, 4 and 7 days after hormone injection.

Table 12. Changes in sperm characteristic thawed cryopreserved sperm that was collected from male Murray cod at different times following injection of hormone to enhance spermiation (Values = mean ± S.E).

Parameter	Thawed				
	Pre-injection	Post-injection			Sign
		2 days	4 days	7 days	
Motility (%)	3.17 ± 1.28	7.56 ± 2.35	21.39 ± 8.93	13.73 ± 5.19	0.0479
VCL	93.37 ± 23.08	58.48 ± 1.96	62.59 ± 6.32	76.02 ± 16.63	0.2518
VAP	49.92 ± 7.06	40.37 ± 1.8	51.1 ± 7.54	47.01 ± 4.37	0.3242
VSL	34.8 ± 12.02	13.46 ± 1.04	17.63 ± 4.16	14.93 ± 2.87	0.0857
LIN	0.61 ± 0.17	0.34 ± 0.03	0.34 ± 0.03	0.31 ± 0.03	0.0827
WOB	0.61 ± 0.13	0.69 ± 0.02	0.81 ± 0.05	0.67 ± 0.07	0.4604
PROG	154.43 ± 55.35	83.78 ± 7.87	96.64 ± 1.62	118.47 ± 37.2	0.393
BCF	7.93 ± 1.73	5.64 ± 0.39	5.25 ± 0.38	7.22 ± 1.41	0.3057

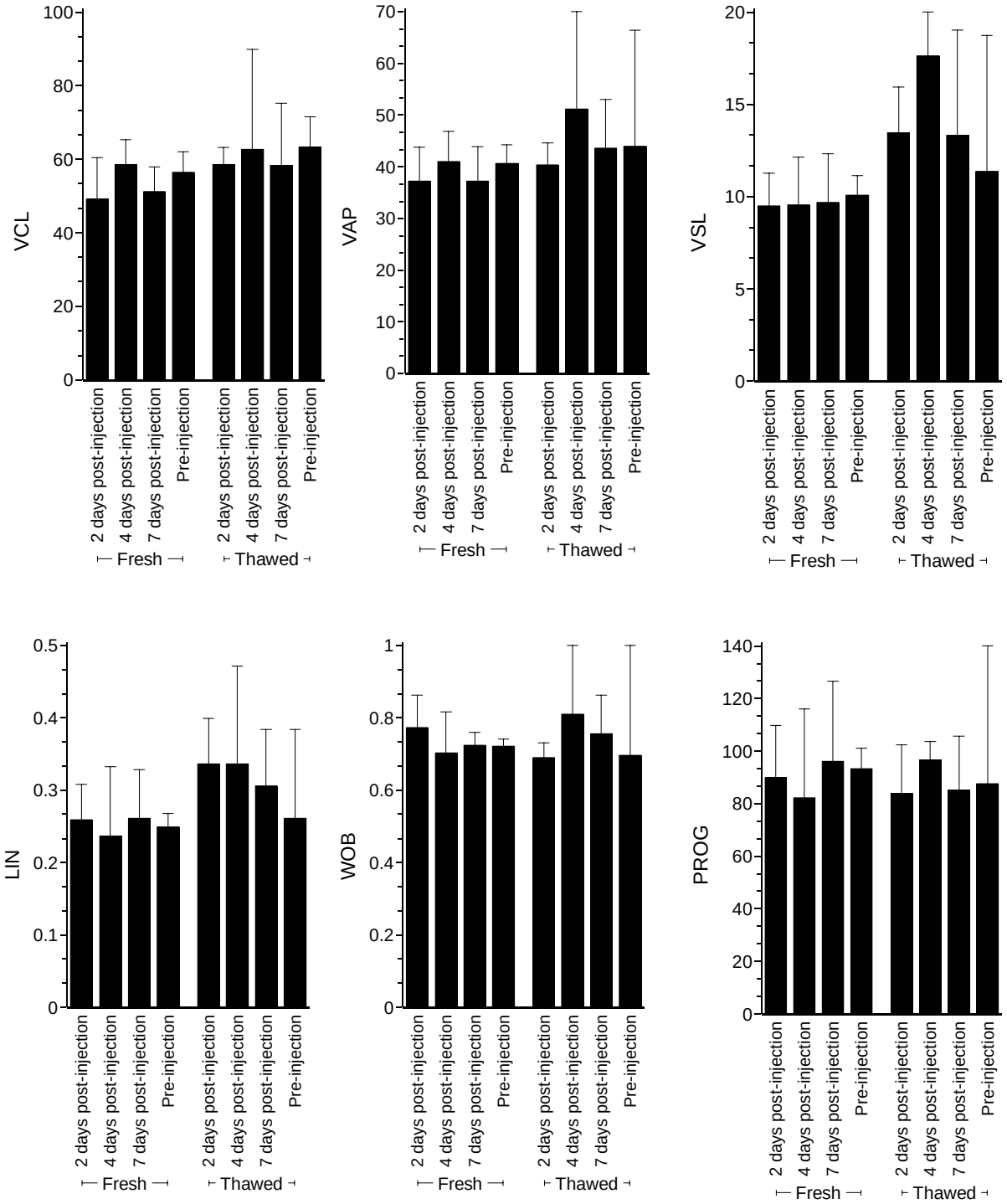

Figure 21. CASA analysis of fresh and thawed Murray cod sperm. VCL = Velocity curvilinear (μm/s). VAP = Velocity average path (μm/s). VSL = Velocity straight line (μm/s). LIN = Linearity (VSL/VAP). WOB = Wobble (VAP/VCL). PROG = Progression.

Fertilisation experiments using cryopreserved sperm

Nine sperm cryopreservation trials were conducted during 2010/11 season, and hatching of larvae was observed in four trials. In eggs fertilised by thawed sperm (that had been frozen in liquid nitrogen for up to 2–30 days), the 24 h egg viability ranged from 51% to 96% (mean 74% ± 5% s.e.) of the viabilities for eggs fertilised with fresh sperm.

The hatch rate in eggs fertilised by thawed sperm ranged from 0% to 27% (mean 15% ± 5% s.e.) of the hatch rates of eggs fertilised with fresh sperm.

Broodstock diet and hormone treatment

Diet treatment

Broodstock

The effects that diet has on the quality of gametes produced by broodstock and subsequent spawning success are summarised in Table 13.

While diet did not affect the growth of broodstock, diet did significantly affect the condition of female broodstock. The condition of females fed the diet formulated specifically for Murray cod by Deakin University (DU; 1.87) was significantly greater than that of females fed the commercially available diet formulated for barramundi (Rid; 1.72). Females fed DU had a significantly higher proportion of oocytes at the S6 stage of development and a lower proportion of resorbing oocytes than did females fed Rid (Table 13).

Oocytes were not significantly different in size, between treatments. Diet did influence the size of eggs striped from mature females. Eggs stripped from fish fed DU were significantly larger and had a higher proportion of cloudy/dirty marks present than did oocytes of females fed Rid. Females fed DU had a higher relative fecundity (2,356 eggs/kg) compared to females fed Rid (1,685 eggs/kg), though this difference was not significant (P=0.0838). Fertilisation and hatch rates of eggs were not significantly different between diets.

A significantly higher proportion of male broodstock fed DU (0.82) were running ripe than for males fed Rid (0.63). Diet had no significant effect on sperm density, motility or other sperm assessments measures (Table 13).

Oocytes from fish fed the DU diet were considerably more orange-brown in colour compared to fish fed the RD diet (control diet) (Figure 22).

The protein content of eggs from fish fed DU was significantly higher than for eggs of fish fed Rid, yet protein content of the diets was not different (Table 14). The mineral content of the diets fed to fish was significantly different for four of the five minerals measured. Diet had no significant effect on the mineral content of the eggs (Table 14).

The Lipid composition of the diets was significantly different (Table 15). The DU diet was higher in n-3 PUFA, n-3 LC-PUFA and n-3/n-6 PUFA, but lower in n-6 PUFA. These significant differences were also reflected in the composition of eggs of fish fed the diets (Table 15).

The amino acid composition of the diets was significantly different (Table 16). Both the total free amino acid and quantitative amino acid pool was significant greater in the DU diet (FAA = 10.12 mg/g, QAA = 372 mg/g) than in the Ridley diet (FAA = 8.44 mg/g, QAA = 350 mg/g).

There were significant differences in the free amino acid content of eggs derived from fish fed with DU diet and those of fish fed with Rid diet. Interestingly these differences did not correspond with the differences in diet formulation. There were no significant differences detected in the quantitative amino acid composition of eggs derived fish fed with the different diets (Table 16). In all cases where a significant difference was found, the free amino acid content was higher in eggs derived from fish fed the Rid diet.

Results of proximate and fatty acid composition analyses of diets and eggs were not available at the time of preparing this report.

Diet had no significant effect on the fertilisation rate, hatch rate, size of larvae upon hatch and larval survival (Table 13).

Hormone treatment

The effects of implants of the hormone Ovaplant diet on the reproductive condition of broodstock, gamete quality and spawning success are summarised in Table 17.

It was also noted that on 7 and 8 December 2011, 5 and 6 days after initial hormone implantation, large numbers of eggs were observed on the filtration screens (2,105 g of eggs, 197,500 eggs) of the RAS (water temperature 15 °C). Eggs did not appear to be fertilised, and there was no indication as to which females shed eggs.

Fish were examined for spawning readiness 16 to 39 days (mean 26 days) after the implantation of the hormone pellets. At this time, many of the implanted fish had a substantially higher proportion of oocytes that were underdeveloped or resorbing. Consequently, there was a significant difference in the proportion of females that were injected (P=0.0001), with just 24% of implanted fish being injected compared to 69% of non-implanted fish (Table 17).

The proportion of implanted fish that ovulated after being induced by a hormone injection was also significantly lower (P=0.0131), with just 50% of implanted fish ovulating compared to 90% of the non-implanted fish.

Mature oocytes (S6 max and min diameter and diameter), ovulated eggs (diameter) and water-hardened eggs from implanted broodstock were significantly smaller than those derived from non-implanted fish (Table 17).

Neither fertilisation rate nor hatch rate of eggs were significantly different between treatments.

Spawning without hormone induction

Three females examined during the spawning seasons had ovulated eggs present at initial examination. The eggs were immediately stripped from these females and fertilised. The number of eggs stripped, egg size and hatch rates are presented in Table 18 and Figure 23.

Hatch rate was positively correlated with fertilisation rate, week of spawning season, and negatively correlated with the proportion of eggs that were coalesced at stripping.

Broodstock condition was positively correlated with the age of the broodstock.

Analysis of spawning data matrix

Data from 1,170 spawning events were analysed, to identify the significant correlations between gamete quality indices, spawning evaluation parameters, and measures of spawning success (Table 19, Figure 23, Figure 24). These analyses found:

- Broodstock condition was positively correlated with fish age (Figure 23a)
- Mean oocyte diameter (sampled at the time of injection) increased with both broodstock condition and broodstock weight (Figure 23a and b).
- The proportion of S5 oocytes in samples collected at the time of spawning declined over the spawning season (Figure 23 d)
- The proportion of stripped eggs that were coalesced increased with centigrade degree hours (CTH) (Figure 23e)
- Spawning following hormone injection was positively correlated with proportion of S6 oocytes in samples collected at the time of hormone injection (Figure 23f).
- Relative fecundity increased with both fish age and proportion of S6 oocytes (Figure 23g and h).

Hatch rate was:

- Positively correlated with:
  - Fertilisation rate
  - Week of spawning season
  - Volume of milt used to fertilise eggs
- Negatively correlated with:
  - The proportion of stripped eggs that were coalesced
  - The proportion of stripped eggs that had cloudy or dirt marks present.

The following equation describes the relationship between the most significant parameters and hatch rate:

Hatch rate = 20.698 - 38.144 Prop. Eggs coalesced + 3.112 Wk of spawning season – 25.129 Prop. Eggs "dirty" (F= 11.16, P<0.0001).

Table 13. Effect of broodstock diet on gamete quality indices, spawning evaluation parameters and measures of spawning success (Values = mean ± s.e.).

Parameter		Diet		
		DU	Rid	Sign.
Broodstock SGR (%/day)	Female	0.04 ± 0.004	0.04 ± 0.004	0.9712
	Male	0.03 ± 0.01	0.02 ± 0.01	0.5873
Broodstock Condition	**Female**	**1.87 ± 0.03**	**1.72 ± 0.02**	**0.0256**
	Male	1.82 ± 0.02	1.73 ± 0.03	0.2595
Oocytes (pre-injection)	S5 prop. of sample	0.19 ± 0.03	0.16 ± 0.02	0.4125
	S6 prop. of sample	**0.7 ± 0.05**	**0.47 ± 0.04**	**0.0038**
	S6 min diameter (mm)	2.77 ± 0.02	2.73 ± 0.03	0.3288
	S6 max diameter (mm)	3.02 ± 0.03	3.0 ± 0.02	0.4988
	S6 diameter	2.87 ± 0.01	2.85 ± 0.02	0.5312
	S6 diameter range (mm)	0.25 ± 0.02	0.28 ± 0.03	0.5225
	Resorbing prop. of sample	**0.17 ± 0.04**	**0.39 ± 0.04**	**0.0004**
Sperm characteristics	Density (cell/µL x 10^6)	69.3 ± 13.5	78.3 ± 15.5	0.6716
	Motility (%)	55.6 ± 7.3	58.4 ± 6.21	0.7641
	VCL	137.1 ± 4.97	138.75 ± 5.4	0.83
	VAP	56.81 ± 1.4	58.09 ± 1.71	0.5849
	VSL	40.33 ± 5.16	43.99 ± 4.93	0.6161
	LIN	0.7 ± 0.08	0.75 ± 0.08	0.6722
	WOB	0.42 ± 0.01	0.43 ± 0.02	0.667
	PROG	198.86 ± 8.82	219.28 ± 8.71	0.1135
	BCF	10.41 ± 0.31	11.33 ± 0.39	0.0861
Prop. females injected		0.75 ± 0.09	0.61 ± 0.02	0.2307
Prop. injected females that ovulated		0.93 ± 0.04	0.86 ± 0.0	0.2
Prop. Males running ripe		**0.82 ± 0.04**	**0.68 ± 0.04**	**0.0319**
Stripped eggs	**Diameter (mm)**	**3.11 ± 0.01**	**3.03 ± 0.01**	**0.0043**
	Prop. coalesced	0.5 ± 0.03	0.4 ± 0.03	0.0980
	Prop. with cloudy/dirt mark	**0.18 ± 0.03**	**0.08 ± 0.03**	**0.0023**
	Relative fecundity (eggs/kg)	2,356 ± 160	1,685 ± 205	0.0838
Water-hardened eggs (diameter mm)		3.24 ± 0.01	3.18 ± 0.02	0.1756
Fertilisation rate (%)		37.9 ± 5.6	61.6 ± 6.4	0.0747
Hatch rate (%)		4.8 ± 2.3	12.3 ± 4.5	0.0599
Hatched larvae (mmTL)		8.24 ± 0.07	8.53 ± 0.05	0.2698
Larval survival (at 21 post hatch) (%)		85.3 ± 4.6	86.6 ± 3.6	0.8267

Where DU = Murray cod broodstcok specific diet formulated by Deakin University
Rid = commercially produced barramundi diet that is routinely fed to Murray cod
Cells shaded blue indicates the DU diet significantly increased the parameter.
Cells shaded yellow indicates the Rid diet significantly increased the parameter

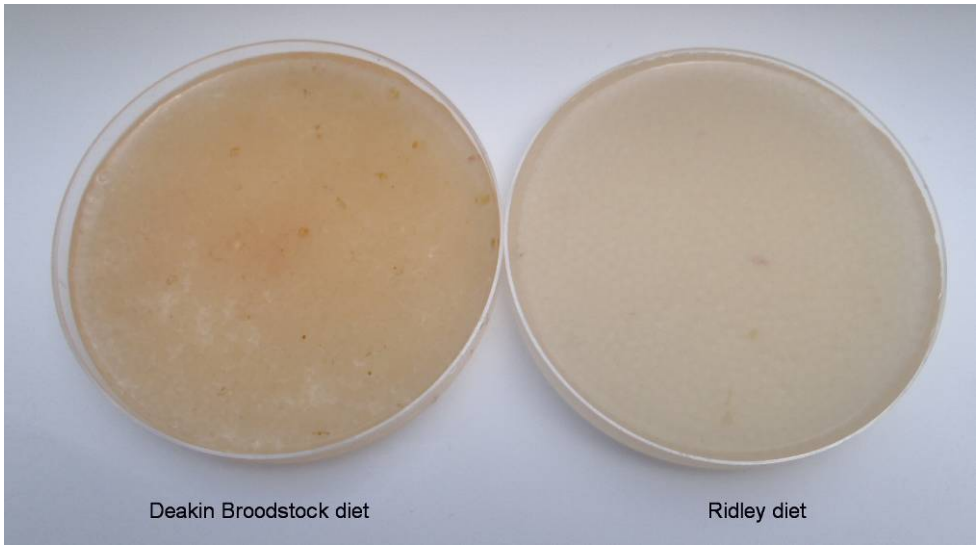

Figure 22. Colour variation in oocytes that have been freshly stripped from broodstock fed different diets.

Table 14. Proximate and mineral composition of two different diets and eggs of broodstock feed the different diets. Data reported as mean ± s.e. (Sign=significance – Bonferroni correction).

Parameter	Diet			Eggs		
	DU	Rid	Sign.	DU	Rid	Sign.
Moisture (% wet wt)	8.62 ± 0.52	6.24 ± 0.16	0.0482	67.3 ± 0.59	72.4 ± 2.1	0.1040
Ash (% dry wt)	10.62 ± 0.07	9.57 ± 0.03	0.0047	1.86 ± 0.24	2.43 ± 0.20	0.1948
Lipid (% dry wt)	14.58 ± 2.45	18.89 ±0.29	0.2226	18.2 ± 0.6	22.0 ± 1.3	0.0650
Protein (% dry wt)	53.0 ± 1.5	48.2 ± 0.4	0.0925	80.0 ± 0.6	75.6 ± 2.10	0.0469
Protein - lipid ratio	3.76 ± 0.73	2.55 ± 0.06	0.2440	4.44 ± 0.17	3.48 ± 0.40	0.0601
Magnesium (mg/kg)	**1600 ± 0**	**1700 ± 0**	**<.0001**	193 ± 21	185 ± 15	0.833
Manganese (mg/kg)	39 ± 1	41 ± 13	0.8922	0.17 ± 0.01	0.26 ± 0.05	0.0689
Phosphorus (mg/kg)	**15,000 ± 0**	**13,000 ± 0**	**<.0001**	2400 ± 168	2400 ± 200	1
Potassium (mg/kg)	**5450 ± 50**	**8950 ± 150**	**0.002**	19 ± 4.93	525 ± 365	0.1599
Selenium (mg/kg)	**1.75 ± 0.05**	**0.93 ± 0.02**	**0.0043**	0.5 ± 0.04	0.32 ± 0.01	0.0548
Zinc (mg/kg)	**205 ± 5**	**145 ± 5**	**0.0136**	16.75 ± 1.11	13.5 ± 0.5	0.127

Where DU = Deakin University Diet. Rid = Ridley diet.

Cells shaded blue indicates that content was significantly higher in the DU diet.
Cells shaded yellow indicates that content was significantly higher in the Rid diet

Table 15. Total fatty acid composition (mg of FA / g of lipid) of two different diets and eggs of broodstock feed the different diets. Data reported as mean ± s.e. (Sign=significance – Bonferroni correction)

Fatty acid	Diets			Eggs		
	DU	Rid	Sign.	DU	Rid	Sign.
12:0	0.53 ± 0.01	0.78 ± 0.01	0.0032	0.07 ± 0.01	0.07 ± 0	0.8878
14:0	20.99 ± 0.98	19.8 ± 0.26	0.3603	5.77 ± 0.29	4.98 ± 0.49	0.2087
14:1n-5	1.2 ± 0.05	1.39 ± 0.04	0.1160	0.24 ± 0.03	0.18 ± 0.01	0.1895
15:0	2.16 ± 0.12	1.99 ± 0.06	0.3327	1.95 ± 0.07	1.73 ± 0.06	0.1248
15:1n-5	0.12 ± 0.07	0.07 ± 0.02	0.5762	0.01 ± 0.01	0 ± 0	0.5415
16:0	149.79 ± 0.83	135.44 ± 24.58	0.6187	89.19 ± 1.08	85.25 ± 6.24	0.3962
16:1n-7	38.84 ± 0.84	40.43 ± 0.28	0.2150	25.41 ± 0.87	18.68 ± 0.42	0.0072
17:0	3.05 ± 0.57	2.71 ± 0.08	0.6122	1.48 ± 0.09	1.5 ± 0.23	0.9380
17:1n-7	0.34 ± 0.04	0.29 ± 0.01	0.3419	0.11 ± 0.02	0.11 ± 0.01	0.9740
18:0	30.51 ± 10.23	48.7 ± 1.26	0.2197	27.5 ± 10.74	45.21 ± 3.38	0.3366
18:1n-9t	1.52 ± 0.9	0.54 ± 0.02	0.2880	6.93 ± 6.77	0.06 ± 0.06	0.5356
18:1n-9	202.52 ± 10.56	231.64 ± 3.32	0.1192	135.08 ± 5.13	132.31 ± 3.08	0.7445
18:1n-7	17.62 ± 0.01	16.66 ± 0.21	0.0467	24.34 ± 0.95	16.85 ± 0.3	0.0064
18:2n-6t	0.8 ± 0.08	0.78 ± 0.02	0.8182	0.76 ± 0.03	0.59 ± 0.02	0.0236
18:2n-6	71.73 ± 1.85	95.05 ± 2.44	0.0168	38.61 ± 1.91	49.79 ± 0.47	0.0177
18:3n-6	1.09 ± 0.13	0.89 ± 0.01	0.2558	1.7 ± 0.16	2.36 ± 0.16	0.0618
18:3n-3	11.02 ± 0.21	12.67 ± 0.36	0.0586	8.54 ± 0.22	8.06 ± 0.63	0.3907
18:4n-3	4.64 ± 0	4.31 ± 0.05	0.0222	1.19 ± 0.1	1.09 ± 0	0.5517
20:0	1.27 ± 0.16	1.46 ± 0.04	0.3759	1.21 ± 0.05	1 ± 0	0.0497
20:1n-11	1.05 ± 0.32	0.75 ± 0.03	0.4496	0.63 ± 0.06	0.63 ± 0.03	0.9702
20:1n-9	2.32 ± 1.69	3.61 ± 0.04	0.5251	0.74 ± 0.53	0.22 ± 0.01	0.5483
20:2n-6	1.23 ± 0.13	0.98 ± 0.02	0.1977	1.91 ± 0.09	2.09 ± 0.11	0.3126
20:3n-6	1.01 ± 0.07	0.85 ± 0.03	0.1704	1.49 ± 0.08	2.06 ± 0.37	0.0887
20:4n-6	4.83 ± 0.15	3.17 ± 0.07	0.0093	7.16 ± 0.24	6.89 ± 0.31	0.5329
20:3n-3	0.48 ± 0.05	0.33 ± 0.01	0.0987	1.09 ± 0.03	0.94 ± 0.07	0.0624
20:4n-3	1.93 ± 0.22	1.55 ± 0.02	0.2229	1.61 ± 0.08	1.63 ± 0.02	0.8697
20:5n-3	35 ± 1.58	29.79 ± 0.08	0.0811	18.24 ± 0.87	19.67 ± 0.27	0.3359
22:0	1.18 ± 0.26	1.1 ± 0.06	0.8016	0.14 ± 0.01	0.18 ± 0.05	0.3377
22:1n-11	0.95 ± 0.95	0.47 ± 0.47	0.6950	0.05 ± 0.03	0 ± 0	0.3400
22:1n-9	1.44 ± 0.85	1.53 ± 0.28	0.9286	0.21 ± 0.01	0.22 ± 0.01	0.5012
22:2n-6	0.15 ± 0.15	0.2 ± 0.07	0.8089	0.18 ± 0.01	0.15 ± 0.01	0.0171
22:4n-6	1.17 ± 0.43	0.67 ± 0.06	0.3642	1.34 ± 0.08	1.14 ± 0.07	0.1976
22:5n-3	6.7 ± 0.43	4.77 ± 0.04	0.0466	17.21 ± 0.37	17.23 ± 0.19	0.9673
22:5n-6	1.76 ± 0.39	0.81 ± 0.02	0.1355	0.15 ± 0.01	1.21 ± 1.04	0.1687
22:6n-3	38.32 ± 2.89	16.41 ± 0.23	0.0170	124.58 ± 4.26	101.31 ± 1.76	0.0229
24:0	0.18 ± 0.18	0.21 ± 0.21	0.9235	0.51 ± 0.04	0.4 ± 0.02	0.1814
24:1n-9	1.09 ± 0.97	1.23 ± 0.01	0.8980	2.01 ± 0.13	1.43 ± 0.05	0.0466

Table 15. Continued

Fatty acid	Diets			Eggs		
	DU	Rid	Sign.	DU	Rid	Sign.
Total	660.52 ± 17.75	684.21 ± 33.95	0.5994	549.28 ± 16.1	527.18 ± 14.66	0.4394
SFA	209.71 ± 9.22	212.44 ± 26.55	0.9313	127.81 ± 10.9	140.32 ± 10.36	0.5152
MUFA	268.98 ± 12.88	298.58 ± 4.13	0.1600	195.74 ± 12.52	170.67 ± 3.18	0.2549
PUFA	181.84 ± 4.34	173.19 ± 3.27	0.2520	225.73 ± 7.84	216.19 ± 1.13	0.4632
n-6 PUFA	83.76 ± 0.62	103.38 ± 2.48	0.0165	53.28 ± 2.52	66.27 ± 0.49	0.0266
n-6 LC-PUFA	10.15 ± 1.02	6.67 ± 0.01	0.0760	12.22 ± 0.47	13.54 ± 0.17	0.1359
n-3 PUFA	98.09 ± 4.96	69.81 ± 0.79	0.0301	172.45 ± 5.33	149.92 ± 0.64	0.0480
n-3 LC-PUFA	82.43 ± 5.16	52.84 ± 0.38	0.0293	162.72 ± 5.05	140.77 ± 1.27	0.0448
n-3/n-6 PUFA	1.17 ± 0.07	0.68 ± 0.01	0.0195	3.25 ± 0.06	2.26 ± 0.01	0.003
n-3/n-6 LC-PUFA	9.71 ± 0.49	10.48 ± 0.09	0.2651	14.14 ± 0.16	11.08 ± 0.19	0.003

Where DU = Deakin University Diet. Rid = Ridley diet.

Cells shaded blue indicates the lipid content was significantly higher in the DU diet.
Cells shaded yellow indicates the lipid content was significantly higher in the Rid diet

Table 16. Free and quantitative amino acid composition of two different diets and eggs of broodstock feed the different diets. Data reported as mean ± s.e. (Sign=significance – Bonferroni correction).

Amino acid	Diet						Eggs					
	Free Amino acids* (mg/g)			Quantitative Amino acids** (mg/g)			Free Amino acids* (mg/g)			Quantitative Amino acids** (mg/g)		
	DU	Rid	Sign.	DU	Rid	Sign.	DU	Rid	Sign.	DU	Rid	Sign.
Histidine	**3.21 ± 0.01**	**0.83 ± 0.02**	**0.0001**	13.15 ± 0.05	13.35 ± 0.05	0.1056	**0.01 ± 0.003**	**0.03±0.002**	**0.0155**	8.45 ± 0.21	8.1 ± 0.2	0.3592
Taurine	**2.78 ± 0.03**	**0.69 ± 0.01**	**0.0002**					0.009				
Asparagine		0.29 ± 0.004					0.01	0.03				
Serine	**0.11 ± 0**	**0.1 ± 0.002**	**0.0065**	15.9 ± 0	16.45 ± 0.3	0.1588	0.01 ± 0.003	0.01±0.008	0.9204	13.38 ± 0.42	12.95 ± 0.25	0.5454
Glutamine	**0.06 ± 0**	**0.05 ± 0**	**<0.0001**				0.004 ± 0.002	0.003±0.002	0.877			
Arginine	**0.39 ± 0.01**	**1.51 ± 0.03**	**0.0008**	**22.75 ± 0.05**	**24.75 ± 0.05**	**0.0012**	0.03 ± 0.01	0.02±0.006	0.5558	15.13 ± 0.47	15.45 ± 0.55	0.6978
Glycine	**0.19 ± 0.001**	**0.14 ± 0.001**	**0.0006**	**19.6 ± 0**	**20.65 ± 0.2**	**0.0198**	**0.01 ± 0.004**	**0.05±0**	**0.0039**	5.75 ± 0.19	5.45 ± 0.15	0.3624
Aspartic acid	**0.1 ± 0.002**	**0.35 ± 0.003**	**0.0002**	**32.5 ± 0.2**	**36 ± 0.4**	**0.0159**	0.02 ± 0.005	0.02±0.01	0.9403	19.43 ± 0.57	18.45 ± 0.15	0.3185
Glutamic acid	**0.41 ± 0.003**	**0.74 ± 0.005**	**0.0003**	**67 ± 0.1**	**49.7 ± 0.2**	**0.0002**	0.05 ± 0.01	0.09±0.05	0.3139	29.58 ± 0.81	28 ± 0.4	0.2745
Threonine	**0.15 ± 0.001**	**0.1 ± 0.002**	**0.0035**	**15.95 ± 0.05**	**14.7 ± 0**	**0.0016**	0.01 ± 0.005	0.05±0.02	0.0607	12.08 ± 0.36	11.6 ± 0.2	0.4435
Alanine	**0.69 ± 0.004**	**0.42 ±0**	**0.0003**	20.15 ± 0.05	20.7 ± 0.2	0.1165	0.04 ± 0.008	0.07±0.02	0.0708	17.23 ± 0.54	16.5 ± 0.3	0.4325
Proline	**0.17 ± 0.001**	**0.17 ± 0.002**	**0.5918**	**21.3 ± 0.1**	**18.35 ± 0.4**	**0.0149**	0.01 ± 0.003	0.01±0.008	0.8664	14.03 ± 0.35	12.9 ± 0.5	0.1361
Ornithine	**0.06 ± 0.001**	**0.03 ± 0**	**0.0003**				**0.02 ± 0.002**	**0.04±0.005**	**0.0432**	2.6 ± 0.1		0.2533
Cysteine				**3.75 ± 0.05**	**3.2 ± 0.1**	**0.0389**					2.4 ± 0	
Cystine							0.002 ± 0	0.01±0.006	0.047			
Lysine	0.35 ± 0.003	0.36 ± 0.001	0.1083	**28.6 ± 0**	**27.15 ± 0.05**	**0.0012**	0.12 ± 0.05	0.23±0.04	0.2292	20.38 ± 0.63	19.7 ± 0.3	0.5228
Tyrosine	**0.15 ± 0.002**	**0.14 ± 0.003**	**0.0316**	**11.4 ± 0**	**9.4 ± 0.4**	**0.0377**	0.02 ± 0.005	0.05±0.02	0.0566	9.25 ± 0.25	8.75 ± 0.35	0.3156
Methionine	**0.31 ± 0.003**	**1.77 ± 0.05**	**0.001**	**11 ± 0**	**6.8 ± 0.1**	**0.0006**	0.09 ± 0.03	0.24±0.13	0.2083	6.83 ± 0.2	6.55 ± 0.15	0.4332
Valine	**0.25 ± 0.003**	**0.18 ± 0.003**	**0.0029**	**20.3 ± 0.1**	**21.9 ± 0.2**	**0.019**	**0.03 ± 0.01**	**0.15±0.01**	**0.0026**	16.58 ± 0.48	15.85 ± 0.35	0.3865
Isoleucine	**0.17 ± 0.001**	**0.13 ± 0.003**	**0.0046**	**17 ± 0.1**	**12.2 ± 0.1**	**0.0009**	**0.03 ± 0.009**	**0.13±0.01**	**0.0032**	14.4 ± 0.43	13.8 ± 0.3	0.4216
Leucine	**0.35 ± 0.002**	**0.24 ± 0.01**	**0.0051**	**29.4 ± 0.1**	**31.75 ± 0.05**	**0.0023**	**0.05 ± 0.02**	**0.18±0.04**	**0.0185**	23.4 ± 0.7	22.45 ± 0.45	0.4289
Phenylalanine	**0.17 ± 0.002**	**0.15 ± 0.003**	**0.0342**	**17.55 ± 0.05**	**19.2 ± 0.1**	**0.0046**	**0.02 ± 0.006**	**0.07±0.02**	**0.0344**	8.68 ± 0.26	8.25 ± 0.25	0.3669
Tryptophan	**0.06 ± 0.001**	**0.07 ± 0.001**	**0.0342**	4.4 ± 0.3	4.2 ± 0.4	0.7278	**0.01 ± 0.003**	**0.02±0.002**	**0.0477**	2.45 ± 0.13	2.4 ±0.2	0.8407
TOTAL_AA	**10.12 ± 0.08**	**8.44 ± 0.01**	**0.0023**	**372 ± 1**	**350 ± 0.3**	**0.0019**	**0.59 ± 0.18**	**1.48±0.24**	**0.0465**	240 ± 6.99	230 ± 3.95	0.4044

* = Calculation based on free amino acid molecular weight. ** = Calculation based on amino acid residue mass in protein (molecular weight minus H_2O); DU = Deakin University Diet. Rid = Ridley diet. Cells shaded blue indicates the amino acid content was significantly higher in the DU diet. Cells shaded yellow indicates the amino acid content was significantly higher in the Rid diet

Table 17. Effect of broodstock hormone pre-treatment (implanted with Ovaplant or not) on gamete quality indices, spawning evaluation parameters and measures of spawning success (Values = mean ± s.e.).

Parameter		Hormone Pre-treatment		
		Implanted	Not implanted	Sign.
Broodstock SGR (%/day)	- Female	-0.03 ± 0.06	0.04 ± 0	0.2916
Broodstock Condition	- Female	1.84 ± 0.04	1.78 ± 0.02	0.7157
Oocytes (pre-injection)	S5 prop. of sample	0.32 ± 0.07	0.17 ± 0.02	0.0595
	S6 prop. of sample	0.35 ± 0.08	0.57 ± 0.03	0.3583
	S6 min diameter (mm)	**2.45 ± 0.09**	**2.75 ± 0.02**	**0.0395**
	S6 max diameter (mm)	**2.8 ± 0.09**	**3.01 ± 0.02**	**0.0314**
	S6 diameter	**2.73 ± 0.04**	**2.86 ± 0.01**	**0.0433**
	S6 diameter range (mm)	0.35 ± 0.06	0.26 ± 0.02	0.4078
	Resorbing prop. of sample	0.43 ± 0.07	0.29 ± 0.03	0.5664
Prop. females injected		**0.24 ± 0.05**	**0.69 ± 0.05**	**0.0001**
Prop. injected females that ovulated		**0.5 ± 0.14**	**0.9 ± 0.02**	**0.0131**
Stripped Eggs	**Diameter (mm)**	**2.90 ± 0.04**	**3.07 ± 0.01**	**0.0023**
	Proportion coalesced	0.36 ± 0.08	0.45 ± 0.02	0.5384
	Proportion with cloudy/dirt mark	0.06 ± 0.03	0.13 ± 0.02	0.5898
	Relative fecundity (eggs/kg)	1308 ± 321	2039 ± 135	0.5236
Water-hardened eggs (diameter mm)		**2.96 ± 0.05**	**3.21 ± 0.01**	**0.0063**
Fertilisation rate (%)		69.3 ± 9.5	49.1 ± 4.5	0.5516
Hatch rate (%)		26.1 ± 18.7	8.3 ± 2.5	0.4486
Hatched larvae (mmTL)		7.76 ± 0.07	8.39 ± 0.05	0.1182

Cells shaded yellow indicated the parameter was significantly higher in the non-implanted fish

Table 18. Spawning without hormone induction in tank-reared Murray cod broodstock.

Female Tag number	Year	No. Eggs stripped	Egg size range (mm)	Hatch rate (%)
6731449	Dec 2008	6,470	2.9–3.3	58
000680ABE0	Nov 2011	600	2.7–3.0	0
000682C9F0	Nov 2011	15,200	2.9–3.2	79

Table 19. Relationships between various gamete quality indices, spawning evaluation parameters, and measures of spawning success.

Parameter A	Parameter B	Pearson Correlation coefficients		Regression	
		Correlation	Sign.		
Broodstock Condition	Fish age (years)	0.51558	<0.0001	1.2569 + 0.0845 Fish age (Adj. R^2 = 0.2649)	Figure 23a
Mean oocyte diameter (mm)	Fish weight (g)	0.38476	<0.0001	2.4785 + 0.000051* fish weight (Adj. R^2 = 0.1457)	Figure 23b
	Broodstock Condition	0.26602	<0.0001	2.2278 + 0.2708 Condition (Adj. R^2 = 0.0682)	Figure 23c
Prop. S5 oocytes	Week of spawning season	-0.23416	<0.0001	0.2964 - 0.026719 Wk of spawning season (Adj. R^2 = 0.0522	Figure 23d
Prop. Eggs coalesced	Centigrade Degree Hours (CTH)	0.53062	0.0161	-4.7470 + 0.0051 CTH. (Adj. R^2 = 0.2416	Figure 23e
Spawned	Prop. S6 oocytes	0.23805	0.0002	0.6632 + 0.3376 Prop. S6 oocytes (Adj. R^2 = 0.0528)	Figure 23f
Relative Fecundity (eggs/kg)	Fish age (years)	0.15377	0.0171	1309.088 + 108.7241 fish age (Adj. R^2 = 0.0195)	Figure 23g
	Prop. S6 oocytes	0.28177	<0.0001	1060.357 + 1258.937 Prop. S6 oocytes (Adj. R^2 = 0.0751)	Figure 23h
Hatch rate	Week of spawning season	0.1604	0.00097	2.8996 + 1.8156 Wk of spawning season (Adj. R^2 = 0.0219)	Figure 24a
	Prop. of eggs coalesced	-0.35978	<0.0001	29.6516 - 40.5513 Prop. Eggs coalesced (Adj. R^2 = 0.1223)	Figure 24b
	Prop. Eggs "dirty"	-0.28091	0.0016	13.9715 - 36.6069 Prop. Eggs dirty (Adj. R^2 = 0.0714)	Figure 24c
	Milt volume (mL)	0.21262	0.009	4.0081 + 3.6616 Milt volume (Adj. R^2 = 0.0388)	Figure 24d
	Fertilisation rate (%)	0.56617	<0.0001	-3.597157 + 0.305137 fertilisation rate (Adj. R^2 = 0.3163)	Figure 24e

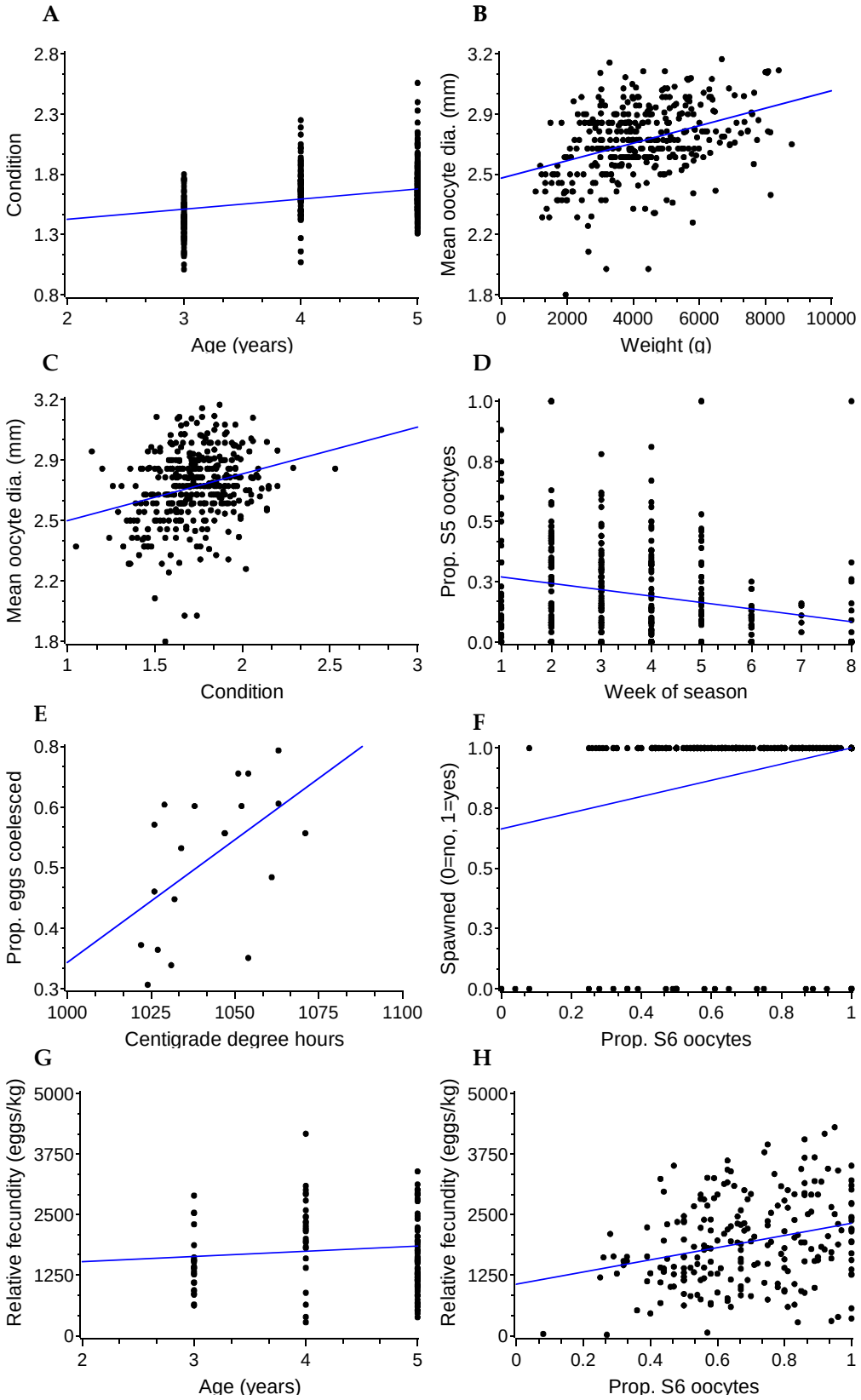

Figure 23. Relationships between various gamete quality indices, spawning evaluation parameters, and measures of spawning success.

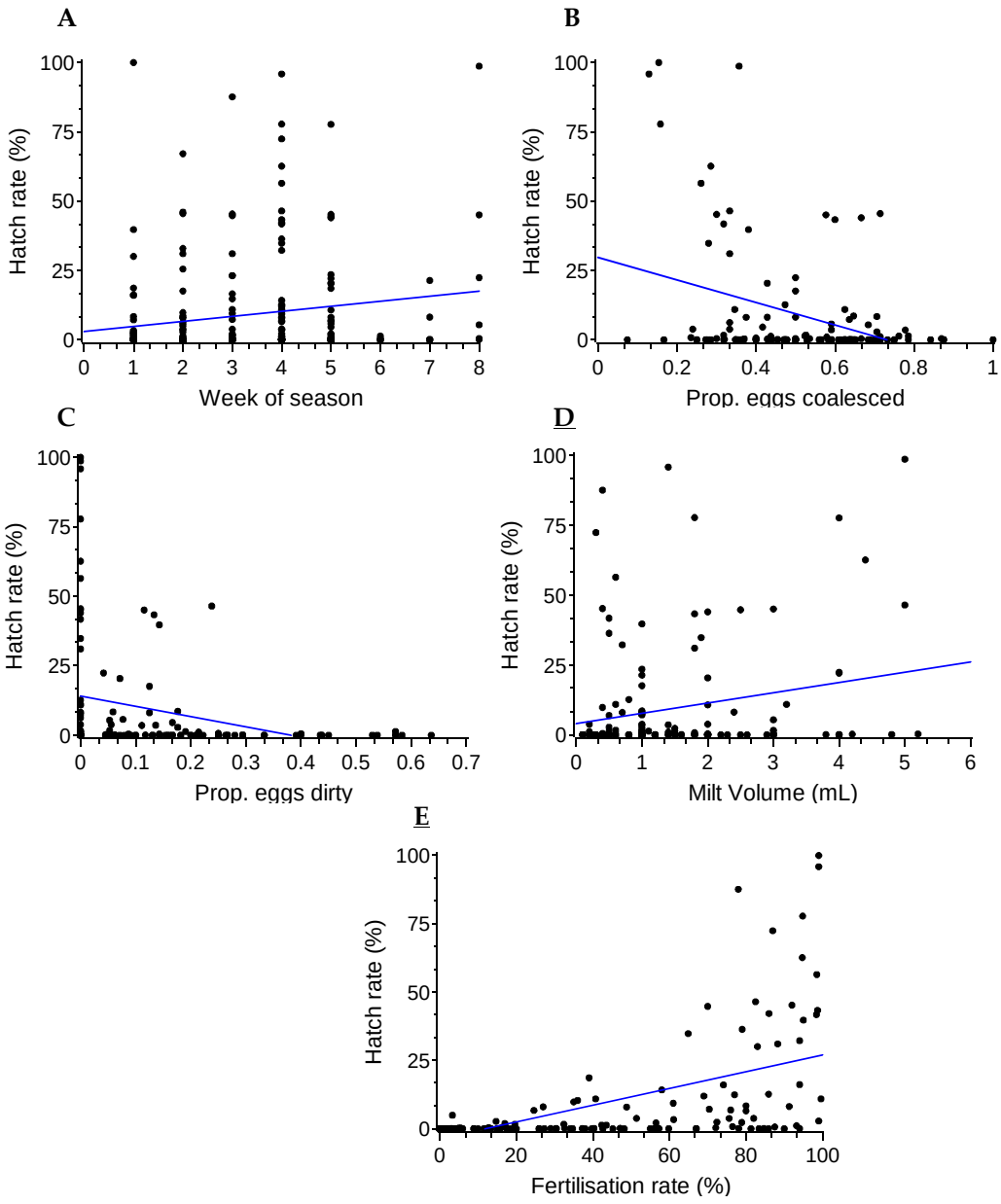

Figure 24. Relationships between hatch rate and various gamete quality indices, spawning evaluation parameters, and measures of spawning success.

Discussion

Comparison between pond-matured and tank-matured broodstock

Tank-matured Murray cod broodstock were readily induced to spawn using conventional hormone treatments with 79–100 % of injected fish ovulating and being stripped each year. This rate of spawning was better than for pond-matured trout cod that were induced to spawn using hormone treatments (Ingram and Rimmer 1992). The high level of spawning indicates that tank-matured Murray cod are reaching final oocyte maturation (FOM), the developmental stage where hormone induction techniques have the greatest influence on ovulation (De Silva et al. 2007).

The fertilisation rate and hatch rate of tank-matured Murray cod were substantially lower than for either pond-matured trout cod or Murray cod. This suggests that even though Murray cod are maturing in tanks, the conditions the fish experience in the tanks are affecting gamete quality and spawning success. Factors that have been implicated in having negative impacts on the quality of gametes produced by tank-reared fish include:

- The environment (e.g. inappropriate temperature, water quality or photoperiod)
- Behavioural factors (e.g. Stress from constant interaction with other fish in tanks)
- Husbandry (e.g. disturbance associated with cleaning tanks, inaccurate timing of hormone induction and stripping)
- Diet (see Bromage and Roberts 1995, Izquierdo et al. 2001, Soso et al. 2008, Mylonas et al. 2010, Callan et al. 2012).

Broodstock nutrition and feeding has been shown to greatly improve not only egg and sperm quality but also seedstock production (Izquierdo et al. 2001). Newman et al. (2008a) suggested that functional abnormalities during secondary oocyte growth may contribute to poor egg fertility in Murray cod broodstock.

This study identified differences in the chemical composition of eggs obtained from pond-matured and tank-matured fish, including differences in the composition of macronutrients of key fatty acids (n-6 and n-3 LC-PUFA). The major differences recorded in total fatty acid and total amino composition were also manifest in the individual lipid and amino acid classes. Differences observed in the chemical composition of eggs obtained from pond-matured and tank-matured fish are likely to be in part due to the diet of the fish. Fish held in the ponds were fed a diet of live prey (fish and crustaceans) whereas fish held in tanks were fed on a commercial pelted diet only. Broodstock fed on `natural diets' often produce better quality eggs of than those fed formulated commercial diets (Brooks et al. 1997).

Previous research has shown that nutrition of broodstock may affect the quality of eggs and larvae. 'Swollen yolk-sac syndrome' (SYSS) is a condition that causes significant mortality in the eggs and newly hatched larvae of Murray cod. SYSS is thought to be caused by nutritional deficiencies in broodstock, which have arisen possibly as a result of the cumulative effects of a poor or limited diet (Gunasekera et al. 1998). Eggs affected by SYSS had significantly lower amounts of free amino acid (FAA) (Gunasekera et al. 1998). Nguyen et al. (2011) showed that viable cobia (*Rachycentron canadumviable*) eggs had significantly higher protein and amino acid contents than non-viable eggs. Successful embryonic development was shown to be dependent on the balance of amino acids present in the egg (Fyhn and Serigstad 1987, Fyhn 1989).

The lipid and fatty acid composition of broodstock diets is a major factor affecting the successful reproduction and survival of offspring (Izquierdo et al. 2001). Fatty acid composition of the diet has also been important for spawning Macquarie perch (*Macquaria australasica*) in captivity. A comparative study of wild and captive-held broodstock showed that the oocytes of the captive-held Macquarie perch were lower in n-6 fatty acids (esp. 20:4n-6) & therefore had higher n-3 to n-6 fatty acid ratios than did oocytes from wild fish (Sheikh-Eldin et al. 1995, Sheikh-Eldin et al. 1996). The low level of n-6 (esp. 20:4n-6) in the diet of tank-reared fish may be responsible for lack of response to hormone-induced spawning owing to the lack of precursors for synthesis of prostaglandins (Ako et al. 1994, Bell et al. 1995).

Studies have shown that the administration of vitamins to fish via broodstock and larval diets have both identified and corrected vitamin decencies in fish (Waagbø, 2010). In this study the concentrations of vitamins in the eggs were too low to be detected and so no comparisons could be made about the relative levels of vitamins in eggs derived from tank-and pond-matured fish.

During this project, trials were conducted to address some of the factors affecting spawning in captive-held Murray cod. Although considerable progress occurred in spawning tank-matured fish (see Figure 13), fertilisation and hatch rates were still lower than observed in pond- matured fish, which suggests that further improvements can be made.

Effect of broodstock diet on spawning

Broodstock diet can have a large effect on egg quality and spawning success in fish (Watanabe 1989, Brooks et al. 1997, Izquierdo et al. 2001, Callan et al. 2012), yet there are no commercially available diets that are formulated specifically for Murray cod broodstock.

Development of the DU broodstock diet aimed to provide a diet that would improve spawning in captive, tank-matured Murray cod. The DU diet was higher in n-3 PUFA, n-3 LC-PUFA and n-3/n-6 PUFA, but lower in n-6 PUFA. These significant differences were reflected in the composition of eggs of fish fed the diets.

Despite significant differences in the amino acid composition of the two diets tested in this study, the amino acid composition of eggs derived from broodstock fed the different diets only differed in a small number of free amino acids. No differences in composition of quantitative amino acids were found. This result may indicate that the amino acid requirements of the eggs were being met by the diets and that excess amino acids are not assimilated by the eggs during development.

Female broodstock fed the DU diet had a higher condition, more mature (stage S6) oocytes, fewer resorbing oocytes, slightly larger eggs and a higher relative fecundity than did broodstock fed Rid. These results suggest that the DU diet enhanced the development of ovaries and oocytes to a greater extent than the Rid diet. However, diet had no effect of fertilisation rates and hatch rates.

Diet had an effect on spermiation. A higher proportion of the males fed the DU diet were running ripe than were males fed the Rid diet.

Production of broodstock specific diets is costly and although the current study suggested some improvements to broodstock quality, hatch rates were not improved. These results suggest that factors other than diet may be affecting spawning success in tank-matured Murray cod broodstock.

Effect of hormone treatment on spawning

Hormone implants, such as Ovaplant, have been used extensively to enhance maturation and improve spawning in captive-held broodstock (Lee *et al.* 1986, Crim *et al.* 1988, Mylonas *et al.* 1996, Mylonas and Zohar 2001, Ingram *et al.* 2005c). In this study, Ovaplant was not effective in improving spawning of tank-reared Murray cod broodstock.

Several weeks after implantation of female Murray cod with Ovaplant, many of the females had a substantially higher proportion of oocytes that were underdeveloped or resorbing. Consequently, fewer implanted fish were injected with HCG. These results suggest that Ovaplant induced at least partial ovulation in many fish.

Some fish may have attempted to spawn or simply shed eggs into the broodstock tanks, as indicated by large numbers of eggs in the RAS filters 5–6 days after implantation while others that could not, or would not, spawn in the broodstock tanks, commenced resorbing overripe oocytes. This may have been due to Ovaplant being implanted too late in the oocyte development cycle.

Interestingly, some of the implanted fish examined towards the end of the spawning season had oocytes samples dominated by S6 stage oocytes. This may have been a second batch of eggs matured from S2 oocytes advanced during the period following implantation.

Previous studies have also identified two distinct batches of vitellogenic oocytes (S5 and S6 stage) in mature Murray cod ovaries, suggesting a capacity for multiple spawns (Rowland 1998b, Newman et al. 2007). Indeed, Murray cod have been shown to spawn more than once in a breeding season. Rourke et al. (2009) used DNA to identify females in captive broodstock that spawned twice in a season, and the second spawning of which tended to be smaller than the

first. Newman et al. (2007) re-induced spawning in post-spawned Murray cod demonstrating the ability of these fish to recruit a secondary batch of post-vitellogenic follicles within weeks of their initial spawn.

During the present study, three females had ovulated in the broodstock tanks without intervention with hormone treatments. The eggs of these females were stripped and fertilised, and produced high hatch rates (58% and 79%) in two spawns.

These results suggest that Murray cod broodstock reared intensively in tanks under artificial conditions and diets have the potential to mature and ovulate unassisted. Continued breeding of Murray cod in captivity using domesticated lines over multiple generations, such as in a selective breeding program, is likely to see improvements in spawning of this species in captivity.

Sperm evaluation and cryopreservation

This study showed that sperm density in Murray cod ranged between 1.2 to 122 x 10^6 per μL (mean 30 x 10^6 per μL). In other studies, sperm density in various marine and freshwater fish has been reported between 2 - >100 x 10^9 per μL (Ciereszko and Dabrowski 1993, Cosson et al. 2008). Apart from an initial decline in the first 2 days following the injection of Murray cod broodstock with a hormone to enhance spermiation, sperm density appeared to be maintained for up to 7 days after injection.

The volume of milt used to fertilise eggs in the current study ranged from 0.1–5.2 mL. The optimal amount of milt (and sperm) required to maximise fertilisation success of Murray cod eggs is unknown. The amount of milt used in this study was dictated more by the volume of eggs stripped, and the amount of milt that could be stripped from specific males (especially where full-sib crosses were being undertaken for selective breeding purposes). Sperm-to-egg ratio can affect fertilisation success, yet it is not clear what the optimal ratio for fertilising Murray cod eggs is. In the current study sperm-to-egg ratios ranged from 2.5-172.5x10^6:1 (mean 26.9x10^6:1).

Butts et al. (2009) found that fertilisation success in Atlantic cod (*Gadus morhua*) significantly decreases below a sperm to egg ratio of 1×10^5:1. Fertilisation success in turbot (*Scophthallmus maximus*) was best at sperm to egg ratios above 6×10^3:1 (Suquet et al. 1995).

Further, polyspermy, which occurs when more than one spermatozoa merges with the sperm entry site on the egg, is not desirable as polyspermic embryos are not viable and die during embryonic development (Urbányi et al. 2009). Further work is required to determine the optimal sperm-to-egg ratio for fertilising Murray cod eggs, and to determine at what ratios polyspermy occurs.

This study showed that motility of sperm declined dramatically in the minutes following activation in freshwater. In order to overcome limitations associated with the short viability of sperm, changes were made to the techniques used to fertilise Murray cod eggs. Prior to this study, sperm stripped from males was collected in a syringe before eggs were stripped from females. The new method was developed to reduce the time between stripping sperm and fertilising eggs. In the new method, ovulated eggs are first stripped from the female, then milt is stripped from the male and added to the eggs within 30 seconds of being stripped from the male.

Sperm motility declined towards the end of the season. Decreases in sperm motility over the spawning season have been observed in some species and not in others (Belova 1981, Suquet et al. 2005, Alavi et al. 2008). Some studies have also shown that sperm velocity decreases over time (Alavi et al. 2008). Velocity may be due to ATP content of sperm, or sperm flagella length. It has been noted that sperm with the higher velocity fertilised a greater proportion of eggs (Gage et al. 2004).

CASA values observed for Murray cod sperm were generally similar to observations on other fish species using the ImageJ Opensource CASA Plugin (e.g. see Wilson-Leedy and Ingermann 2007, Sanches et al. 2010). In contrast, in the south American catfish (*Rhamdia quelen*) VSL (46.5), and consequently LIN (0.78) (Sanches et al. 2010), were considerably greater than in Murray cod.

Many spermatozoa of Murray cod examined in this study had a looped flagella (Figure 19), which appears to be atypical for normal fish spermatozoa. Whether or not this abnormal morphology affected the sperm's motility and ability to fertilise an egg is not known.

Sperm deformities may be associated with functional deficiencies and cause reduced motility and fertilisation ability (Rurangwa et al. 1998, Rurangwa et al. 2004). Spermatozoa

exposed to toxicants, for example, may possess broken tails (Van Look and Kime 2003) or lack flagella at sexual maturity (McAllister and Kime 2003). More detailed morphological and biochemical studies of Murray cod spermatozoa are required to shed light on this subject.

Fertilisation experiments using cryopreserved sperm

Results from fertilisation experiments using thawed sperm indicated that, although larvae were hatched, hatch rates were substantially lower than for eggs fertilised with fresh sperm. These differences may be attributed to a range of factors including the effects of cryoprotectants on spermatozoa. This study showed that thawed sperm had significantly lower motility, while other characteristics such as velocity were unaffected.

Although the cryopreservation method developed by Daly et al. (2008) was effective in freezing Murray cod sperm, further work is required to refine the current technique to improve sperm survival and viability post-thawing.

Refinement of sperm cryopreservation methods for Murray cod will also provide a basis for improved management of genetic resources in breeding programs for other species used in aquaculture, stock enhancement and conservation.

A sperm bank was established and samples of sperm from Murray cod broodstock held at Snobs Creek as part of the selective breeding program. In addition, sperm from Macquarie perch (*Macquaria australasica*), a threatened species, was successfully frozen using the D-Sorbitol method of Daly et al. (2008).

Growth rate and age at maturity

The present study showed that growth of Murray cod broodstock was not only highly variable between individuals but, when comparing regression coefficients, males grew significantly faster than females. Neither Gooley (1992) nor Rowland (1998a) found a significant difference in growth rates of males and females. However, Anderson et al. (1992) found that the growth of males was slower than for females, and suggested that differences between sexes may be apparent in larger and older fish only.

Although more specific research is required to validate differences in growth rates between sexes under aquaculture conditions, these results suggest that production of faster growing fish for aquaculture may be achieved by producing all-male stock. That approach has been used for other farmed species, for example, YY male Nile tilapia are used as broodstock in YY x XX crosses to produce genetically male tilapia for aquaculture. In this case, the use of all YY males improves yields compared to mixed sex tilapia of the same strain or sex reversed male tilapia. This is achieved by overcoming culture problems with early sexual maturation and uncontrolled reproduction leading to overpopulation (Mair et al. 1995, Mair et al. 1997).

Studies show that the age and size at maturity in wild Murray cod varies considerably. At three years of age a few males are mature, while at four years of age most males and females are mature (Rowland 1998b). Yet, in Lake Charlegrark, females mature at approximately 6 years of age and 2 kg, and males at 3–4 years of age and 700 g (Gooley 1992).

Captive broodstock appeared to have matured at a younger age and smaller size compared to wild fish. In this study, most (97%) 3 year old males were running ripe while nearly half (43.5%) of the 3 year old females and most (78%) 4 year old females were mature and were injected to induce spawning, of which >78% ovulated. Newman et al. (2008a) found some 2 year old and all 3 year old tank-reared Murray cod were mature.

Early maturation in tank-reared fish may be attributed to altered environmental conditions experienced in the hatchery. Broodstock were held indoors in RAS and although water temperature follows a typical seasonal cycle, the use of heaters to temper low winter temperatures can hasten maturity.

The ability to mature Murray cod earlier in captivity may be a benefit to the selective breeding program for Murray cod, as this will reduce the generation time and thereby increase the genetic gain of selected traits.

Sex ratio and hermaphrodism

The population of captive Murray cod had a sex ratio that was biased towards more females than males (♀ : ♂ 1.91:1). Retention of fish in the breeding program up to this time had been random.

Information on sex ratios in Murray cod populations has not been widely reported. In wild populations of Murray cod, sex ratios of ♀ : ♂ 0.72–0.85:1 (Anderson et al. 1992), and ♀ : ♂ 1.3–1.7:1 (Rowland 1998a) have been

reported. Lyon et al. (2012) reported more females than males (♀ : ♂ 2.5:1) in a population of trout cod stocked from hatchery bred fish.

Sex determination in many fish species can be influenced by environmental and genotypic factors (Devlin and Nagahama 2002, Penman and Piferrer 2008). Temperature factors seem to be the main environmental factor affecting sex in fish. The critical period for temperature to affect sex ratios is before and during the onset of the histological gonadal sex differentiation in fry or eggs (Baroiller and D'Cotta 2001). Since all broodstock used in this study had been incubated and reared under hatchery conditions, the sex ratio of these fish may have been influenced by temperature regimes in the hatchery. Alternatively, survival rates during grow-out may have been different between sexes.

The occurrence of hermaphrodites in fish is not uncommon having been observed in at least 25 families of fish (Devlin and Nagahama 2002, Patzner 2007), as well as Murray cod (Gooley et al. 1995). In the histological examination of gonads from 81 fish, Gooley et al. (1995) found three fish (2.39–4.02 years of age) that were macroscopically classified as female with small regions of testicular tissue present in the gonads that were dominated by ovarian tissue.

Various gonadal differentiation types have been described for fish, including species that are purely female or male (i.e. gonochorists), as well as hermaphroditic species that can initially mature either as males (protandrous) or females (protogynous) (Devlin and Nagahama 2002).

The two hermaphroditic Murray cod observed in the present study are classed as synchronous hermaphrodites as they contained functional male and female gonadal tissues at the same time. This is an extremely rare occurring in just a small percentage (0.36%) of the examined fish. The stripped gametes (eggs and sperm) from these hermaphrodite fish were mixed but spawning did not result in hatched larvae.

Spawning data matrix analysis

Preliminary analysis of a data matrix of 1,170 spawning events identified a number of significant correlations between gamete quality indices, spawning evaluation parameters, and spawning success.

The increase in broodstock condition with fish age may reflect the improved nutrition of broodstock that occurred in later years of the project (i.e. use of a specifically formulated broodstock diet).

Increasing age and broodstock condition also corresponded with an increase in mean oocyte diameter (sampled at the time of injection) and relative fecundity. Larger body size often results in the production of larger eggs (e.g. DeMartini 1991). Egg size is also affected or modulated by the nutritional status of the female during ovarian recrudescence (Bromage et al. 1990). Larger eggs usually have a higher yolk content, which may improve larval survival. This study found broodstock diet affected the size of stripped eggs but did not affect the size of oocytes. The presence of mature, stage S6, oocytes also increased the likelihood of spawning in Murray cod following hormone injection.

Hatch rate was positively correlated with fertilisation rate, week of spawning season, and volume of milt used to fertilise eggs, and was negatively correlated with the proportion of stripped eggs that were coalesced and the proportion of stripped eggs that had cloudy or dirt marks present. These latter two variables appear to be linked to latency period (time between injection and spawning) as both increased with increasing centigrade degree hours (CTH).

Highly coalesced eggs with a dirty or cloudy appearance (Figure 5) may represent morphological changes within the egg associated with over ripening (Formacion et al. 1993). Over ripening is one of the most common reasons for poor egg quality in captive broodstock (Brooks et al. 1997). Rowland (1988) found that the time of stripping and fertilising Murray cod eggs strongly affected hatchability. Eggs stripped within 1 h of ovulation or 4–6 h after ovulation had reduced hatch rates.

In this study however, hatch rate was not significantly correlated with latency period or CTH. Never the less the appearance of ovulated eggs (i.e. the presence of coalescing of oil globules, dirty or cloudiness, degree of translucency) may be developed into indicators for assessing the optimal time for stripping ovulated eggs from Murray cod. Apart from appearance, over-ripeness may also be assessed by measuring ovarian pH (Fauvel et al. 1993, Lahnsteiner et al. 2009).

The volume of milt used to fertilise eggs was positively correlated with hatch rate. This study also showed that the volume of milt stripped increased with broodstock weight. A key factor

in determining the relevance of milt volume to hatch rate is the optimal ratio of eggs to sperm required to maximise the fertilisation rate. This ratio cannot be determined from milt volume alone as sperm density varies considerably with milt.

Conclusions

This study described the growth and maturation of Murray cod reared in tanks under controlled conditions. It identified:

- Differences in growth rates between males and females
- A more skewed sex ratio in captive stock
- The incidence of hermaphrodism.

A comparison between pond-matured and tank-matured broodstock showed considerable differences breeding performance between the culture systems. There were considerable variations in oocyte and egg characteristics. In the days following fertilisation, the percent of eggs that remained viable declined at various rates. Most of the mortalities occurred in the first three days following fertilisation. This period may represent a critical stage of embryonic development, which affects survival.

There was a high incidence of sperm with hooked flagella, but the cause of this and its affect on sperm viability is unknown. Although density of Murray cod sperm was measured for the first time, further research is now required to identify sperm-to-egg ratios for maximising the fertilisation rate of Murray cod eggs. Improved understanding of sperm motility in the minutes following activation, resulted in changes to fertilisation methods during spawning of Murray cod. In particular, the time between stripping milt and fertilising eggs was reduced to less than 30 sec.

Freezing sperm in liquid nitrogen then thawing greatly reduced sperm motility, but other characteristics, such as velocity, were unaffected. Fertilisation trials using thawed sperm resulted in hatched larvae, but hatch rates were considerably lower than for eggs fertilised with fresh sperm. Refinement of sperm cryopreservation methods will improve management of breeding programs for aquaculture, stock enhancement and conservation.

A sperm bank has been established at Snobs Creek and samples of sperm from selected Murray cod broodstock, Macquarie perch and trout cod have been frozen using current methods.

A diet specifically formulated for Murray cod broodstock improved the condition of females and slightly increased relative fecundity. Diet had no effect on fertilisation rates and hatch rates.

Hormone treatment using Ovaplant did not improve spawning success in this study. During the project, two female Murray cod ovulated without the intervention with hormone treatments. These eggs were fertilised and had high hatch rates.

These results suggest that intensively reared broodstock have the potential to mature and ovulate unassisted. Continued breeding of Murray cod in captivity using domesticated lines over multiple generations may see improvements in spawning.

Analysis of information for 1,170 spawning events identified a number of significant correlations between gamete quality indices, spawning evaluation parameters, and measures of spawning. Hatch rate was positively correlated with fertilisation rate, week of spawning season, and volume of milt used to fertilise eggs, and was negatively correlated with the proportion of stripped eggs that were coalesced and the proportion of stripped eggs that had cloudy or dirt marks present.

Recommendations

The gamete quality parameters measured during the study have revealed a number of factors that influence hatch rate in Murray cod. Other factors which influence the quality of gametes have been described in other studies (see Brooks et al. 1997, Rurangwa et al. 2004, Bobe and Labbé 2009, Cabrita et al. 2009, Lahnsteiner et al. 2009, Fauvel et al. 2010). The factors that should be investigated in future studies, include:

- Physicochemical parameters of ovarian or coelomic fluid
  Low pH values of ovarian fluid have been associated with post-ovulatory ageing and reduced egg quality (Bobe and Labbé 2009, Fauvel et al. 2010). High protein levels associated with protein leakage from eggs and low pH are indicators of over ripening (Lahnsteiner et al. 2009, Fauvel et al. 2010)

- Weight increase of eggs following fertilisation
  Weight increase of eggs during water-hardening is due to osmosis of water into the perivitelline space from the external environment. This was observed in this study as water-hardened eggs were slightly larger than ovulated eggs (Figure 14a). The percent of egg weight increase during water-hardening provides information on progress of the cortical reaction, concentration of osmotic active compounds and functionality of the chorion (Lahnsteiner et al. 2009)
- Genetic influences and maternal RNAs on oocyte and egg quality (Brooks et al. 1997, Bobe and Labbé 2009)
- Concentrations of metabolites and enzyme activity (Lahnsteiner et al. 2009)
- Hormone concentrations
  Maternal hormones, which have been shown to be passed into oocytes, may play a regulatory role in larvae for growth, development, osmoregulation and other physiological functions prior to the functional development of their own endocrine glands (Lam 1994). Thyroid hormones of maternal origin that are deposited in egg yolk may have significant affect on embryo development (Lam 1994)
- Sperm assessment
  Sperm morphology, plasma membrane composition and functionality, acrosome status, mitochondria status and functionality, metabolic status and DNA integrity (Rurangwa et al. 2004, Cabrita et al. 2009).

Poor husbandry can result in poor reproductive success of cultured fish, and generally there has been little attention paid to the effects of husbandry practices on gamete quality. While this study identified a number of practices that may improve gamete quality (such as changes to methods for fertilising eggs), other factors need to be considered. In captivity, confinement and crowding may affect egg quality. Confinement experienced during the final stages of reproductive development, and periods of acute stress, have been shown to affect endocrinology, which underpins growth and development of ovaries, leading to reduced quality of gametes (Campbell et al. 1994, Brooks et al. 1997, Schreck et al. 2001).

Information presented in this report will assist in improving hatchery production not just for Murray cod but other Maccullochella spp. (trout cod, eastern freshwater cod and Mary River cod) that are produced for stocking purposes.

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References

Ako, H., Tamaru, C.S. and Lee, C.S. (1994). Chemical and physical differences in milkfish (*Chanos chanos*) eggs from natural and hormonally induced spawns. *Aquaculture* 127 (2-3): 157-167.

Alavi, S.M.H., Psenicka, M., Rodina, M., Policar, T. and Linhart, O. (2008). Changes of sperm morphology, volume, density and motility and seminal plasma composition in *Barbus barbus* (Teleostei: Cyprinidae) during the reproductive season. *Aquatic Living Resources* 21: 75-80.

Anderson, J.R., Morison, A.K. and Ray, D.J. (1992). Age and growth of Murray cod, *Maccullochella peelii* (Perciformes: Percichthyidae), in the lower Murray-Darling Basin, Australia, from thin sectioned otoliths. *Australian Journal of Marine and Freshwater Research* 43: 983-1013.

Anon (2010). *Department of Primary Industry Snobs Creek Greenhouse Operations Manual 2010*. DPI Internal Standard Operations Procedure (SOP) Document. 19 pp.

Baroiller, J.F. and D'Cotta, H. (2001). Environment and sex determination in farmed fish. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 130 (4): 399-409.

Bell, J.D., Castell, J.D., Tocher, D.R., McDonald, F.M. and Sargent, J.R. (1995). Effect of different dietary fatty acids: docosahexaenoic acid ratios on phospholipid fatty acid composition and prostaglandin production in juvenile turbot (*Scophrhalmus maximus*). *Fish Physiology & Biochemistry* 14: 139-151.

Belova, N.V. (1981). The ecological and physiological peculiarities of sperm in pond cyprinids. Communication I. Production and ecological and physiological peculiarities of the sperm of cyprinids. *Journal of Ichthyology* 21: 90-102.

Bobe, J. and Labbé, C. (2009). Egg and sperm quality in fish. *General and Comparative Endocrinology* doi: 10.1016/j.ygcen.2009.02.011.

Bromage, N. and Roberts, R.J. eds. (1995). *Broodstock Management and Egg and Larval Quality.*, Blackwell Science Ltd., Oxford.

Bromage, N.R., Hardiman, P., Jones, J., Springate, J. and Bye, V. (1990). Fecundity, egg size and total egg volume differences in 12 stocks of rainbow trout, *Oncorhynchus mykiss Aquaculture and Fisheries Management* 21: 269-284.

Brooks, S., Tyler, C.R. and Sumpter, J.P. (1997). Egg quality in fish: what makes a good egg? . *Reviews in Fish Biology & Fisheries* 7 (4).

Butts, I.A.E., Trippel, E.A. and Litvak, M.K. (2009). The effect of sperm to egg ratio and gamete contact time on fertilization success in Atlantic cod Gadus morhua L. *Aquaculture* 286 (1-2): 89-94.

Cabrita, E., Robles, V. and Herráez, P. (2009). Sperm quality assessment. In: *Methods in Reproductive Aquaculture: Marine and Freshwater Species* (Cabrita, E., Robles, V. and Herráez, P. eds.), pp. 93-147. CRC Press, London.

Cadwallader, P.L. and Gooley, G.J. (1985). *Propagation and Rearing of Murray cod Maccullochella peeli at the Warmwater Fisheries Station Pilot Project Lake Charlegrark*. Fisheries and Wildlife Service, Department of Conservation, Forests and Lands, Melbourne. 189 pp.

Callan, C.K., Laidley, C.W., Forster, I.P., Liu, K.M., Kling, L.J. and Place, A.R. (2012). Examination of broodstock diet effects on egg production and egg quality in flame angelfish (Centropyge loriculus). *Aquaculture Research* 43 (5): 696-705.

Campbell, P.M., Pottinger, T.G. and Sumpter, J.P. (1994). Preliminary evidence that chronic confinement stress reduces the quality of gametes produced by brown and rainbow trout. *Aquaculture* 120: 151-169.

Christie, W.W. (2003). *Lipid Analysis (3rd ed.). Isolation, Separation, Identification and Structural Analysis of Lipids*. The Oily Press, PJ Barnes and Associates, Bridgewater, United Kingdom. 416 pp.

Ciereszko, A. and Dabrowski, K. (1993). Estimation of sperm concentration of rainbow trout, whitefish and yellow perch using a spectrophotometric technique *Aquaculture* 109 (3-4): 367-373

Cosson, J., Groison, A.-L., Suquet, M., Fauvel, C., Dreanno, C. and Billard, R. (2008). Studying sperm motility in marine fish: an overview on the state of the art. *Journal of Applied Ichthyology* 24 (4): 460-486.

Crim, L.W., Sherwood, N.M. and Wilson, C.E. (1988). Sustained hormone release. II. Effectiveness of LHRH analog (LHRHa) administration by either single time injection or cholesterol pellet implantation on plasma gonadotropin levels in a bioassay model fish, the juvenile rainbow trout. *Aquaculture* 74: 87-95.

Daly, J., Galloway, D., Bravington, W., Holland, M. and Ingram, B. (2008). Cryopreservation of sperm from Murray Cod, *Maccullochella peelii peelii*. *Aquaculture* 285: 117-122.

De Silva, S.S., Nguyen, T.T.T. and Ingram, B.A. (2007). Fish reproduction in relation to aquaculture. In: *Fish Reproduction* (Rocha, M., Arukwe, A. and Kapoor, B.G. eds.), pp. 535-575. Science Publishers, Enfield, New Hampshire, USA.

DeMartini, E.E. (1991). Annual variations in fecundity, egg size, and the gonadal and somatic conditions of queenfish *Seriphus politus* (Sciaenidae). *Fishery Bulletin* 89: 918.

Devlin, R.H. and Nagahama, Y. (2002). Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences [Review]. *Aquaculture* 208 (3-4): 191-364.

Douglas, J.W., Gooley, G.J. and Ingram, B.A. (1994). *Trout Cod, Maccullochella macquariensis (Cuvier) (Pisces: Percichthyidae), Resource Handbook and Research and Recovery Plan*. Department of Conservation and Natural Resources, Melbourne. 99 pp.

Fauvel, C., Omnes, M.H., Suquet, M. and Normant, Y. (1993). Reliable assessment of overripening in Turbot (*Scophthalmus maximus*) by a simple pH measurement. *Aquaculture* 117 (1-2): 107-113.

Fauvel, C., Suquet, M. and Cosson, J. (2010). Evaluation of fish sperm quality. *Journal of Applied Ichthyology* 26 (5): 636-643.

Folch, J., Lees, M. and Sloane Stanley, G.H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *Journal of Biological Chemistry* 226(a): 497-509.

Formacion, M.J., Hori, R. and Lam, T.J. (1993). Overripening of ovulated eggs in goldfish I. Morphological changes. *Aquaculture* 114: 155-168.

Francis, D.S., Turchini, G.M., Jones, P.L. and De Silva, S.S. (2007). Dietary lipid source modulates in vivo fatty acid metabolism in the freshwater fish, murray cod (Maccullochella peelii peelii). *Journal of Agricultural & Food Chemistry* 55 (4): 1582-1591.

Fyhn, H.J. (1989). First feeding of marine fish larvae - are free amino-acids the source of energy. *Aquaculture* 80: 111-120.

Fyhn, H.J. and Serigstad, B. (1987). Free amino acids as energy substrate in developing eggs and larvae of the cod Gadus morhua. *Marine Biology* 96: 335-341.

Gage, M.J.G., MacFarlane, C.P. and Yeates, S. (2004). Spermatozoal traits and sperm competition in Atlantic salmon: relative sperm velocity is the primary determinant of fertilization success. . *Curr. Biol.* 14: 44-47.

Gjedrem, T. ed. (2005). *Selection and Breeding Programs in Aquaculture,* Springer, Dordrecht, The Netherlands. 364 pp.

Gjedrem, T. (2012). Genetic improvement for the development of efficient global aquaculture: A personal opinion review. *Aquaculture*.

Gooley, G.J. (1992). Validation of the use of otoliths to determine the age and growth of Murray cod, *Maccullochella peelii* (Mitchell) (Percichthyidae), in Lake Charlegrark, western Victoria. *Australian Journal of Marine and Freshwater Research* 43: 1091-1102.

Gooley, G.J., Anderson, T.A. and Appleford, P. (1995). Aspects of the reproductive cycle and gonadal development of Murray cod, Maccullochella peelii peelii (Mitchell) (Pisces: Percichthyidae), in Lake Charlegrark and adjacent farm ponds, Victoria, Australia. *Marine and Freshwater Research* 46: 723-728.

Gunasekera, R.M., Gooley, G.J. and De Silva, S.S. (1998). Characterisation of 'swollen yolk-sac syndrome' in the Australian freshwater fish Murray cod, *Maccullochella peelii peelii*, and associated nutritional implications for large scale aquaculture. *Aquaculture* 169 (1-2): 69-85.

Ho, H.K., Rourke, M.L., Bravington, W., McPartlan, H. and Ingram, B.A. (2008). Genetic and reproduction technologies for enhanced

aquaculture and fisheries management of Murray cod. *Aquaculture Asia* 13 (1 (Jan-Mar)): 15-21.

Ingram, B. and Larkin, B. (2000). Murray cod aquaculture - current information and current status. In: *Murray Cod Aquaculture A Potential Industry for the New Millennium. Proceedings of a Workshop (18th January 2000, Eildon, Vic.)* (Ingram, B.A. ed.), pp. 4-16. Marine and Freshwater Resources Institute, Alexandra.

Ingram, B.A. and De Silva, S.S. eds. (2004). *Development of Intensive Commercial Aquaculture Production Technology for Murray cod. Final Report to the Fisheries Research and Development Corporation (Project No. 1999/328).* Primary Industries Research Victoria, DPI, Alexandra, Victoria, Australia. 202 pp.

Ingram, B.A., De Silva, S.S. and Gooley, G.J. (2005a). The Australian Murray cod - A new candidate for intensive production systems. *World Aquaculture* 36 (3): 37-43 and 69.

Ingram, B.A., Gavine, F. and Lawson, P. (2005b). *Fish Health Management Guidelines for Farmed Murray Cod*. Fisheries Victoria, Technical Report Series No. 32, Alexandra, VIC. 56 pp.

Ingram, B.A., Hayes, B. and Rourke, M.L. (2011). Impacts of stock enhancement strategies on the effective population size of Murray cod, *Maccullochella peelii*, a threatened Australian fish. *Fisheries Management & Ecology* 18: 467-481.

Ingram, B.A., McPartlan, H., Hayes, B., King Ho, H.K., Bravington, W., Chamberlain, A., Nheu, J. and Guthridge, K. (2008). *Final Report for Project 08307: Water Innovation Program - Future Aquaculture, genetic tools for a changing environment*. Department of Primary Industries, Melbourne. In press pp.

Ingram, B.A., McPartlan, H., Ho, H.K., Rourke, M. and Bravington, W. (2007). *Final Report for Project 05194: High Value Aquaculture in Sustainable Rural Landscapes*. Our Rural Landscape. Sustainable Development Through Innovation. Department of Primary Industries, Melbourne. 27 pp.

Ingram, B.A. and Rimmer, M.A. (1992). Induced breeding and larval rearing of the endangered Australian freshwater fish trout cod, *Maccullochella macquariensis* (Cuvier) (Percichthyidae). *Aquaculture and Fisheries Management* 24: 7-17.

Ingram, B.A., Sungan, S., Gooley, G.J., Sim, Y.S., Tinggi, D. and De Silva, S.S. (2005c). Induced spawning, larval development and rearing of two indigenous Malaysian mahseer, *Tor tambroides* and *T. douronensis*. *Aquaculture Research* 36: 1001-1014.

Izquierdo, M.S., Fernandez-Palacios, H. and Tacon, A.G.J. (2001). Effect of broodstock nutrition on reproductive performance of fish. *Aquaculture* 197: 25-42.

Lahnsteiner, F., Soares, F., Ribeiro, L. and Dinis, M.T. (2009). Egg quality determination in teleost fish. In: *Methods in Reproductive Aquaculture: Marine and Freshwater Species* (Cabrita, E., Robles, V. and Herráez, P. eds.), pp. 149-180. CRC Press, London.

Lam, T.J. (1994). Hormones and egg/larvae quality in fish. *Journal of the World Aquaculture Society* 25(1): 2-12.

Lee, C.-S., Tamaru, C.S. and Kelley, C.D. (1986). Technique for making chronic-release LHRH-a and 17x-Methyltestosterone pellets for intramuscular implantation in fishes. *Aquaculture* 59: 161-168.

Lyon, J.P., Todd, C., Nicol, S.J., McDonald, A., Stoessel, D., Ingram, B.A., Barker, R.J. and Bradshaw, C.J.A. (2012). Reintroduction success of threatened Australian trout cod (*Maccullochella macquariensis*) based on growth and reproduction. *Marine and Freshwater Research* 63: In press.

Mair, G.C., Abucay, J.S., Beardmore, J.A. and Skibinski, D.O.F. (1995). Growth performance of genetically male tilapia (GMT) derived from YY-males in *Oreochromis niloticus* L.: on-station comparisons with mixed sex and sex reversed male populations. *Aquaculture* 137: 313-322.

Mair, G.C., Abucay, J.S., Skibinski, D.O.F., Abella, T.A. and Beardmore, J.A. (1997). Genetic manipulation of sex ratio for the large-scale production of all-male tilapia, *Oreochromis niloticus*. *canadian Journal of Fisheries & Aquatic Sciences* 54: 396-404.

McAllister, B.G. and Kime, D.E. (2003). Early life exposure to environmental levels of the aromatase inhibitor tributyltin causes masculinisation and irreversible sperm damage in zebrafish (*Danio rerio*). *Aquatic Toxicology* 65: 309-316.

Mylonas, C.C., Fostier, A. and Zanuy, S. (2010). Broodstock management and hormonal manipulations of fish reproduction. *General and comparative endocrinology*.

Mylonas, C.C., Magnus, Y., Gissis, A., Klebanov, Y. and Zohar, Y. (1996). Application of controlled-release, GnRHa-delivery systems in commercial production of white bass x striped bass hybrids (sunshine bass), using captive broodstocks. *Aquaculture* 140 (3): 265-280.

Mylonas, C.C. and Zohar, Y. (2001). Use of GnRHa-delivery systems for the control of reproduction in fish. *Reviews in Fish Biology & Fisheries* 10 (4): 463-491.

Newman, D.M., Jones, P.L. and Ingram, B.A. (2007). Temporal dynamics of oocyte development, plasma sex steroids and somatic energy reserves during seasonal ovarian maturation in captive Murray cod *Maccullochella peelii peelii*. *Comparative Biochemistry & Physiology Part A* 148 (4): 876-887.

Newman, D.M., Jones, P.L. and Ingram, B.A. (2008a). Age-related changes in ovarian characteristics, plasma sex steroids and fertility during pubertal development in captive female Murray cod *Maccullochella peelii peelii*. *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology* 150 (4): 444-451.

Newman, D.M., Jones, P.L. and Ingram, B.A. (2008b). Sexing accuracy and indicators of maturation status in captive Murray cod Maccullochella peelii peelii using non-invasive ultrasonic imagery. *Aquaculture* 279 (1-4): 113-119.

Newman, D.M., Jones, P.L. and Ingram, B.A. (2010). Advanced ovarian development of Murray cod *Maccullochella peelii peelii* via phase-shifted photoperiod and two temperature regimes. *Aquaculture* 310: 206-212.

Nguyen, H.Q., Reinertsen, H., Rustad, T., Tran, T.M. and KjÃ¸rsvik, E. (2011). Evaluation of egg quality in broodstock cobia Rachycentron canadum L. *Aquaculture Research*.

Núñez, J. and Duponchelle, F. (2008). Towards a universal scale to assess sexual maturation and related life history traits in oviparous teleost fishes. *Fish Physiology and Biochemistry* DOI 10.1007/s10695-008-9241-2.

Palmeri, G., Turchini, G.M. and De Silva, S.S. (2007). Lipid characterisation and distribution in the fillet of farmed Australian native fish, Murray cod (*Maccullochella peelii peelii*). *Food Chemistry* 102: 796-807.

Patzner, R.A. (2007). Reproductive Strategies of Fish. In: *Fish Reproduction* (Rocha, M., Arukwe, A. and Kapoor, B.G. eds.), pp. 311-350. Science Publishers, Enfield, New Hampshire, USA.

Penman, D.J. and Piferrer, F. (2008). Fish gonadogenesis. part I: Genetic and environmental mechanisms of sex determination. *Reviews in Fisheries Science* 16 (SUPPL.1): 14-32.

Rourke, M.L., McPartlan, H.C., Ingram, B.A. and Taylor, A.C. (2009). Polygamy and low effective population size in a captive Murray cod (*Maccullochella peelii peelii*) population: genetic implications for wild restocking programs. *Marine and Freshwater Research* 60 (8): 873-883.

Rowland, S.J. (1983). Spawning of the Australian freshwater fish Murray cod, *Maccullochella peeli* (Mitchell), in earthen ponds. *Journal of Fish Biology* 2: 525-534.

Rowland, S.J. (1988). Hormone-induced spawning of the Australian freshwater fish Murray Cod, *Maccullochella peeli* (Mitchell) (Percichthyidae). *Aquaculture* 70: 371-389.

Rowland, S.J. (1998a). Age and growth of the Australian freshwater fish Murray cod, *Maccullochella peelii peelii*. *Proceedings of the Linnean Society of New South Wales* 120: 163-180.

Rowland, S.J. (1998b). Aspects of the reproductive biology of Murray cod, *Maccullochella peelii peelii*. *Proceedings of the Linnean Society of New South Wales* 120: 147-162.

Rurangwa, E., Kime, D.E., Ollevier, F. and Nash, J.P. (2004). The measurement of sperm motility and factors affecting sperm quality in cultured fish [Review]. *Aquaculture* 234 (1-4): 1-28.

Rurangwa, E., Roelants, I., Huyskens, G., Ebrahimi, M., Kime, D.E. and Ollevier, F. (1998). The minimum effective spermatozoa: egg ratio for artificial insemination and the effects of mercury on sperm motility and fertilisation ability in *Clarias gariepinus*. *Journal of Fish Biology* 53: 402-413.

Sanches, E.A., Bombardelli, R.A., Marcos, R.M., Neumann, G., De Toledo, C.P.R. and Romagosa, E. (2010). Sperm motility of *Rhamdia quelen* studied using computer-assisted analysis by

open-source software. *Aquaculture Research* 42 (1): 153-156.

Schreck, C.B., Contreras-Sanchez, W. and Fitzpatrick, M.S. (2001). Effects of stress on fish reproduction, gamete quality, and progeny. *Aquaculture* 197 (1-4): 3-24.

Sheikh-Eldin, M., De Silva, S.S., Anderson, T.A. and Gooley, G. (1995). Physical characteristics and proximate composition of oocytes, liver and muscle of wild caught and tank-reared Macquarie perch. *Aquaculture International* 3: 172-185.

Sheikh-Eldin, M., De Silva, S.S., Anderson, T.A. and Gooley, G.J. (1996). Comparison of fatty acid composition of body muscle, liver, mature oocytes and diets of wild and captive Macquarie perch, *Macquaria australasica* broodfish. *Aquaculture* 144: 201-216.

Soso, A.B., Gil Barcellos, L.J., Ranzani-paiva, M.J., Kreutz, L.C., Quevedo, R.M., Lima, M., Bolognesi da Silva, L., Ritter, F., Bedin, A.C. and Finco, J.A. (2008). The effects of stressful broodstock handling on hormonal profiles and reproductive performance of rhamdia quelen (Quoy & Gaimard) females. *Journal of the World Aquaculture Society* 39 (6): 835-841.

Suquet, M., Billard, R., Cosson, J., Normant, Y. and Fauvel, C. (1995). Artificial insemination in turbot (*Scophthallmus maximus*): determination of the optimal sperm to egg ratio and time of gamete contact. *Aquaculture [Aquaculture]* 133: 83-90.

Suquet, M., Rouxel, C., Cosson, J., Severe, A., Quemener, L. and Fauvel, C. (2005). Changes in atlantic cod (*Gadus morhua*) sperm quality with time. In: *Larvi 2005, European Aquaculture Society Special Publication* (C.I., H., Van Stappen, G., Wille, M. and Sorgeloss, P. eds.), pp. 503-505

European Aquaculture Society.

Turchini, G.M., Francis, D.S. and De Silva, S.S. (2007). Finishing diets stimulate compensatory growth: results of a study on Murray cod, Maccullochella peelii peelii. *Aquaculture Nutrition* 13 (5): 351-360.

Urbányi, B., Horváth, Á. and Bokar, Z. (2009). Artificial fertilization in aquaculture species: from normal practice to chromosome manipulation. In: *Methods in Reproductive Aquaculture: Marine and Freshwater Species* (Cabrita, E., Robles, V. and Herráez, P. eds.), pp. 183-216. CRC Press, London.

Van Look, K.J.W. and Kime, D.E. (2003). Automated sperm morphology analysis in fishes: the effect of mercury on goldfish sperm. *Journal of Fish Biology* 63: 1020- 1033.

Waagbø¸, R. (2010). Water-soluble vitamins in fish ontogeny. *Aquaculture Research* 41 (5): 733-744.

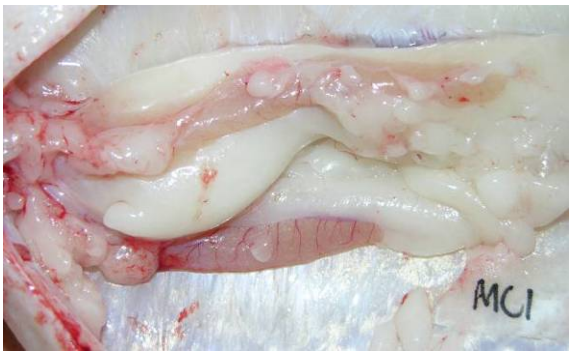
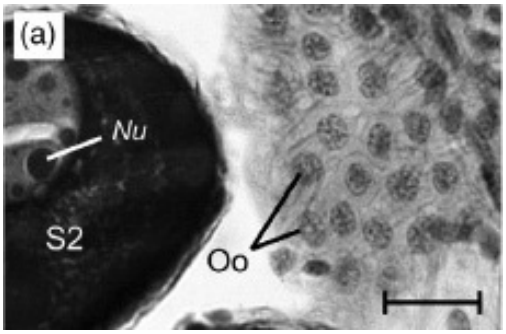
Watanabe, T. (1989). Effect of broodstock diets on reproduction of fish. *Advances in tropical aquaculture*: 77.

Wilson-Leedy, J.G. and Ingermann, R.L. (2007). Development of a novel CASA system based on open source software for characterization of zebrafish sperm motility parameters. *Theriogenology* 67 661-672.

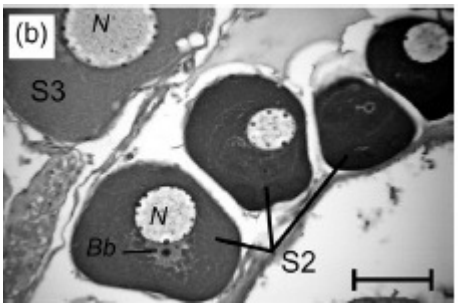
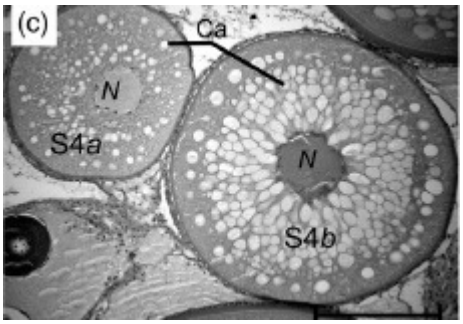
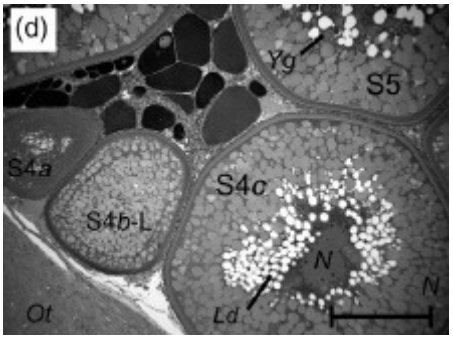
Wyse, L.C. (1973). Artificial spawning of Murray cod. *Aquaculture* 2: 420-432.

Żarski, D., Palińska, K., Targońska, K., Bokor, Z., Kotrik, L., Krejszeff, S., Kupren, K., Horváth, A., Urbányi, B. and Kucharczyk, D. (2011). Oocyte quality indicators in Eurasian perch, Perca fluviatilis L., during reproduction under controlled conditions. *Aquaculture* 313 (1-4): 84-91.

Appendix 1 - Gamete developmental stages of Murray cod

<table>
<tr><th>FEMALES</th><th></th></tr>
<tr><td>IMMATURE
(A) Ovaries small, thin, partly translucent or pale pink. Oocytes invisible to the naked eye. (1–2 years old)
(B) Ovaries rounded, cylindrical, pale pink, translucent, up to 80 mm long, 10 mm wide; sometimes white oocytes (to 1 mm) visible in clear fluid (2 & some 4 year old).</td><td> 
(Photo by Dane Newman)</td></tr>
<tr><td>MATURING
Ovaries much larger, occupying a significant part of the abdominal cavity. Filled with white or yellowish oocytes of different sizes.</td><td></td></tr>
<tr><td>Oogonia (Oo)
Cytoplasm scarce and transparent. Nucleus occupies majority of cell volume.
Diameter: 5–9 (7) µm</td><td> 

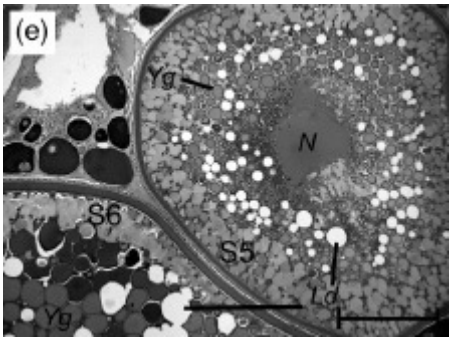
(Photo source: Newman et al. 2007)</td></tr>
<tr><td colspan="2">Chromatin nucleolar (S1)
Narrow ring of moderately basophilic cytoplasm and a nucleus containing a single, large nucleolus.
Diameter: 10–23 (20) µm</td></tr>
</table>

Early perinucleus (S2) Highly basophilic cytoplasm with occasional concentrated areas of differentially-staining Balbiani bodies. Several prominent nucleoli surround the inner-nucleus periphery. *Diameter: 18–111 (68) μm*	 (Photo source: Newman *et al.* 2007)
Late perinucleus (S3) Cytoplasm becomes progressively homogenous and less basophilic. Nucleoli extend further towards the periphery of the nucleus. *Diameter: 94–254 (151) μm*	
Early cortical alveoli (S4a) Developing cortical alveoli appear throughout the cytoplasm and begin to accumulate towards the outer-perimeter of the nucleus. *Diameter: 234–367 (297) μm*	 (Photo source: Newman *et al.* 2007)
Mid-late cortical alveoli (S4b) Cytoplasm becomes acidophilic. Cortical alveoli increase in number and size and form a large circum-nuclear aggregation which progressively extends towards the oocyte periphery. A separate band of alveoli occupy the cytoplasm between the main aggregation and the cell membrane. Developing follicular layer becomes visible during this development stage. *Diameter: 304–869 (557) μm*	
Lipid droplet (S4c) Aggregation of cortical alveoli occupies virtually the entire area between the nucleus and cell membrane. Non-staining lipid droplets appear between alveoli and form a separate aggregation around the external periphery of the nucleus. Follicular envelope becomes increasingly prominent with zona radiata and granulosa cell layers clearly discernable. *Diameter: 737–1,020 (868) μm*	 (Photo source: Newman *et al.* 2007)

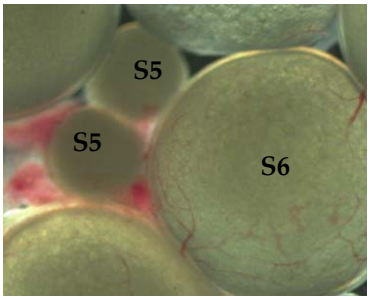
Early vitellogenic (S5)

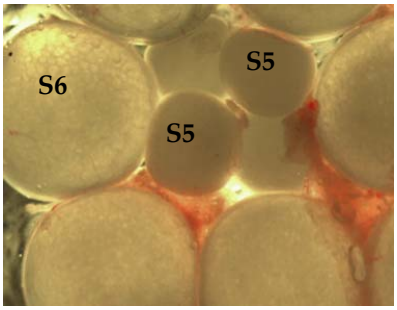
Highly acidophilic yolk granules appear between the cortical alveoli and gradually coalesce to form yolk globules. Yolk globules initially form in close proximity to the lipid droplets but become increasingly prolific and progressively occupy the outer cytoplasm. Lipid droplets are eventually distributed throughout the oocyte and cortical alveoli are displaced towards the oocyte periphery.

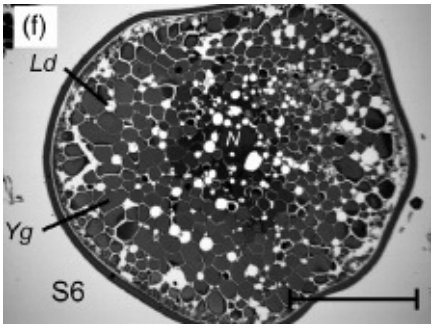
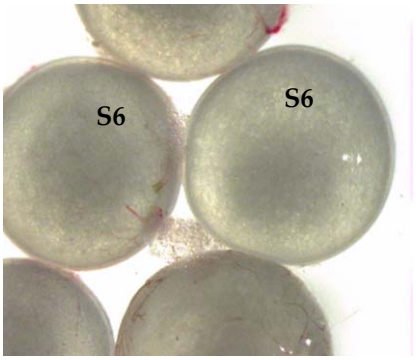
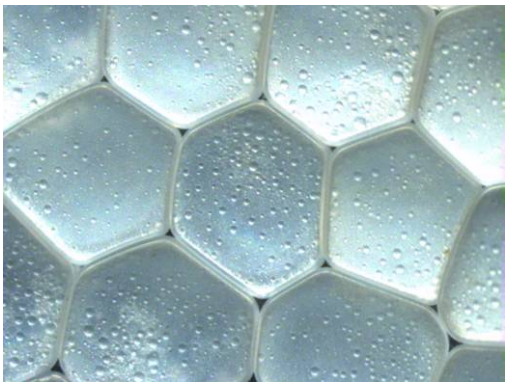
Diameter: 798–2,051 (1,201) µm

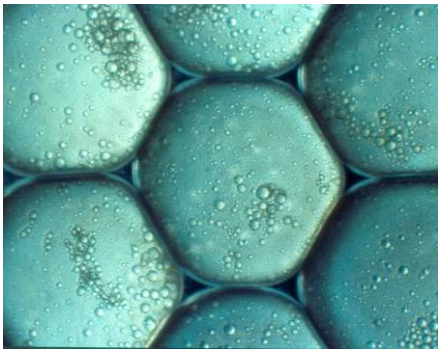
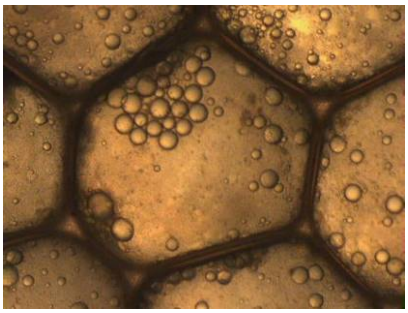


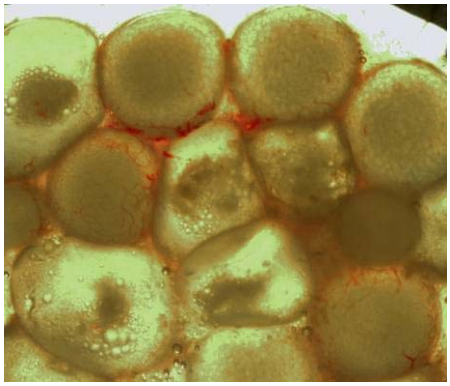
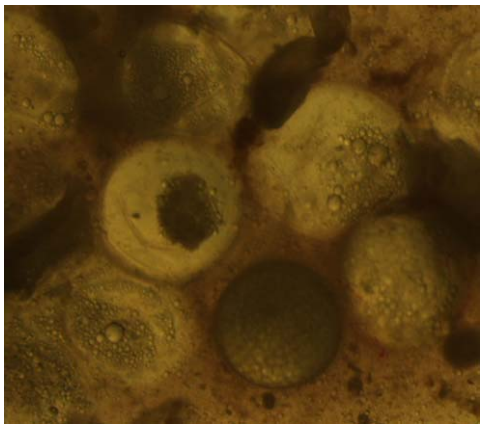
(Photo source: Newman *et al.* 2007)



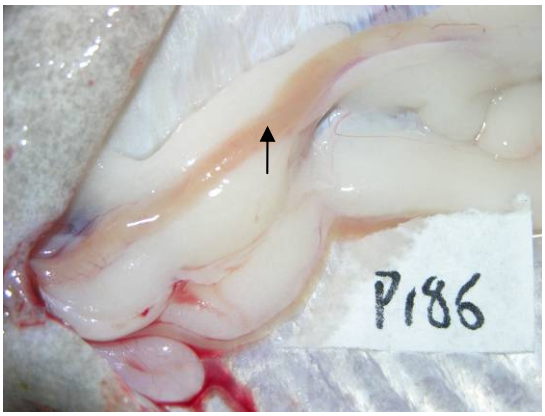


Late vitellogenic (S6) Oocytes opaque/frosty to translucent. Yolk globules are distributed throughout most of the oocyte and cortical alveoli occupy a narrow band between the yolk and follicle membrane. Zona radiata is highly prominent. *Diameter: 1,522–3,084 (2,415) µm*	 (Photo source: Newman *et al.* 2007) 
(V1) Partially ovulated Oocytes partially ovulated, not free in the ovarian cavity. Oocytes translucent (becoming clear), oil globules visible and showing signs of coalescing. Abdomen distended, vent swollen, red/purple. (Snobs Ck Stage 2)	

(V2) Running Ripe Oocytes fully ovulated, (free in the ovarian cavity) and can be expelled with gentle pressure of the fish flanks. Oocytes translucent to clear, oil globules coalesced (fully polarised). Ovaries pinkish/pales red, occupy >1/2 body cavity. Abdomen very distended, vent swollen, red/purple. (Snobs Ck Stage 3)	 
(VI) Spent Ovaries still very large but almost empty, flaccid, often red/dark pink. Some parts of ovaries clear, some small (1mm) white oocytes visible Some remaining ripe oocytes may still be present along with atretic follicles. Abdomen not distended, flabby.	

(Res) Artesia/Resorbed Oocytes clear, flaccid, ovarian fluid may be in higher volume, often bloody with oil globules present as a result of liquefaction of yolk globules followed by progressive fragmentation and degeneration of follicular layer and oocyte structure. (Snobs Ck Stage 4)	 
Resting Oocytes similar to immature ovaries (see above), but are usually larger, wider and pink to dark red in colour. The ovarian wall is also thicker. It distinguishes between a mature and resting female and an immature fish.	

(modified from Snobs Creek Notes, Rowland 1998b, Newman *et al.* 2007 and Núñez and Duponchelle 2008)

MALES	
IMMATURE (A) Testes thin strap-like, pale, translucent threads. (B) In later stage, thickening, opaque.	
MATURING Testes are longer, wider, often of triangular or circular section and whitish to pinkish colour.	 (Photo by Dane Newman)

(V) Running Ripe

Testes full, rounded, completely white. Milt emitted with pressure on the abdomen. Abdomen not distended, vent pale/pinkish and slightly swollen.

Subjective Assessment of expressed milt		
Milt Volume	***Sperm Consistency***	**Sperm Activity**
0 = Nil	**1** = Very watery	**0** = Nil activity
1 = Small amount (drops)	**2** = Watery/milky	**1** = Poor ('jiggly". <10% motile)
2 = "Good" (up to 1 mL)	**3** = Milky	**2** = Fair(10-50% motile)
3 = "Lots" (>1 mL)	**4** = Thick creamy-white	**3** = Good (>50% motile)
		4 = Excellent (100% motile in swirling masses)

(VI) Spent

Testes still large as a running ripe stage, but flaccid, empty-like. Small amount or no milt on pressure.

Resting

Similar to maturing stage

Appendix 2 - Formulation of an experimental Murray cod broodstock diet

Raw materials	g/kg
Fish meal	572
Wheat Gluten	95
Wheat Flour	165
Fish Oil	118
vit+min+others *	50
Expected composition[&]	
PG %	50
LG %	17
Ash %	5
NFE %	22
GE (Mj/kg)	23
**** Vit+Min+others***	g/kg
Ridely vit+min[$]	5
Choline	5
Ethoxyquin	0.2
Vit C	10
Astaxanthin	1
Lecithin	10
Vit E	0.3
beta-glucan	1
selenomethionine[#]	0.3
filler (whatever)	17.2

[#](from Seyeast, Selplex, Alltech, Stamford, Lincolnshire, UK)

[&]Expected composition computed on the following assumptions: Fish Meal: PG 70%, LG 8.5%, Ash 8%; Wheat Flour: PG 12%, LG 1.4%, Ash 3%; Wheat Gluten: PG 83%, LG 4.5%, Ash 2% (raw materials composition can vary from batch to batch)

[$] Ridely "Standard" Min & Vit mix to be included at 3kg/t

Appendix 3 - List of publications and presentations using information and data from recent Murray cod reproduction research

Ingram, B.A. (2009). Culture of juvenile Murray cod, trout cod and Macquarie perch (Percichthyidae) in fertilised earthen ponds. Aquaculture 287: 98-106.

Ingram, B.A. (2010). Aquaculture R&D in Victoria, focusing on the Aquaculture Futures Initiate (AFI). In SARDI Alliance Workshop (January 2010), Adelaide.

Ingram, B.A. (2010). Murray cod Aquaculture Research in Victoria. In Aquaculture Association of Queensland Annual Conference (Hervey Bay 13-14 August 2010), Hervey Bay.

Ingram, B. (2010). A brief history of integrated aquaculture R&D in Victoria. In Integrated Agri-Aquaculture Workshop:"Growing fish in your dam" (17 March 2010) DPI, Mildura.

Ingram, B.A., Ho, H.K., Nguyen, T.T.T., Turchini, G., Bradley, T. and Blume, A. (2012). Current research in Murray cod aquaculture. In Australian Aquaculture Melbourne 2012 (1-4 May 2012, Melbourne).

Ingram, B.A., Rowland, S.J. and Gooley, G.J. (2012). Captive breeding of Australian native fish species for stock enhancement of inland waters - some case studies. In Australian Aquaculture Melbourne 2012 (1-4 May 2012, Melbourne).

Newman, D.M., Jones, P.L. and Ingram, B.A. (2010). Advanced ovarian development of Murray cod *Maccullochella peelii peelii* via phase-shifted photoperiod and two temperature regimes. Aquaculture 310: 206-212.

Olsen, L., Ingram, B.A., Gooley, G.J. and Bailey, M. (2010). Aquaculture in multi water-use farming systems for a changing climate. In Australasian Aquaculture (Hobart 23-26 May 2010).

Rourke, M.L., McPartlan, H.C., Ingram, B.A. and Taylor, A.C. (2009). Polygamy and low effective population size in a captive Murray cod (*Maccullochella peelii peelii*) population: genetic implications for wild restocking programs. Marine and Freshwater Research 60 (8): 873-883.

Turchini, G. (2009). Murray cod Nutrition. In Feed and Water Quality Management Workshop for Open-Water Murray cod Farmers (2 Sep. 2009, Swan Hill), Swan Hill.

Turchini, G.M. (2011). Murray cod open water nutrition. 2009-2011. Final report. Deakin University, Warrnambool. 37 pp.