DEVELOPMENT OF HATCHERY TECHNIQUES FOR THE PRODUCTION OF WA DHUFISH
(Glaucosoma hebraicum)

Challenger TAFE

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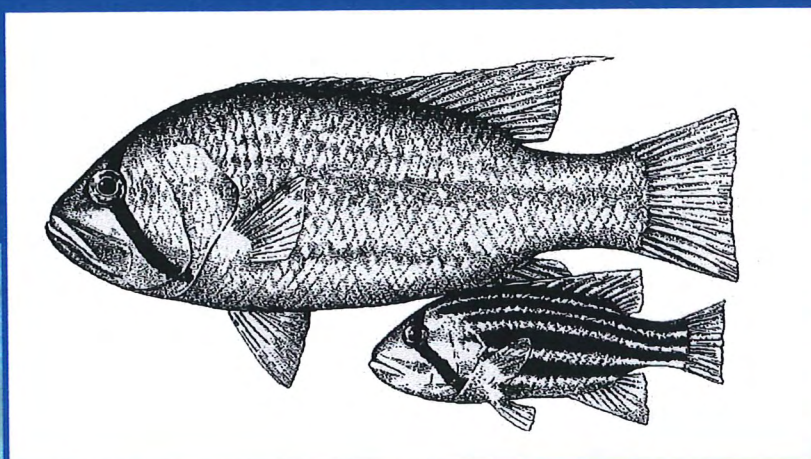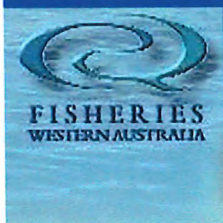
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Chapter 1:   Non-technical summary

The Aquaculture Development Unit of Challenger TAFE has been conducting research to develop aquaculture techniques for dhufish since 1995. In that time significant advances have been made.

1. Techniques for capturing, handling and holding dhufish in captivity have been developed

2. Techniques for obtaining fertilised eggs from both acclimated and wild-caught dhufish have been developed

3. Larvae have been reared successfully

4. Larvae have been successfully weaned to a commercial pellet

5. Hatchery-reared fish have reached sexual maturity

6. A range of fish health issues have been identified and various treatments tested

Although dhufish show potential as an aquaculture species, there are several areas that require attention

1. Egg production is currently insufficient to support a commercial venture. If the hatchery-reared broodstock spawn, this problem will be alleviated.

2. Commercial-scale larviculture techniques must be refined to increase survival and reliability.

3. Nutritional requirements must be understood and a suitable grow-out diet developed

4. A range of health issues must be addressed, including exophthalmia in juveniles and broodstock, "fatty liver" syndrome in juveniles, gill fluke infestation in juveniles and broodstock.

Chapter 2: Introduction

2.1 Background

The Western Australian Maritime Training Centre (WAMTC) is the Fremantle campus of Challenger TAFE, which is managed by the Western Australian Department of Training and Employment (WADTE). During 1992, the Chief Executive Officer of WADTE began implementing a policy of associating TAFE colleges closer to industry. This included the provision of facilities to encourage industry groups to participate in applied research projects and joint ventures. In 1993 funding was allocated to formally establish an Aquaculture Developmental Unit (ADU) at WAMTC. The principal objective of the ADU is to assist with the development of the marine aquaculture industry in Western Australia.

The emphasis of the ADU is on the development of cost-effective production techniques suitable for marine finfish culture. Target species include pink snapper, *Pagrus auratus*, black bream (*Acanthopagrus butcheri*), WA dhufish (*Glaucosoma hebraicum*), estuary cod (*Epinephelus coioides*) and King George whiting (*Sillaginodes punctata*). In addition, investigations are being carried out to develop aquaculture techniques for various species of molluscs, particularly Roe's abalone (*Haliotis roei*) and staircase abalone (*Haliotis scallaris*).

The ADU have been conducting research into the development of aquaculture techniques for dhufish since 1995. This research has been funded by Challenger TAFE and the Fisheries Research and Development Corporation (FRDC) with supplementary funding provided by Fisheries WA and Recfishwest. There have been three successive FRDC projects on the culture of dhufish: Project 95/095 (1year); Project 96/302 (3 years) and 1999/322 (1 year). The success of these projects has led to interest by private investors in funding the project. This document reports on Projects 96/302 and 1999/322. There are five main foci for research to develop culture techniques for WA dhufish: general husbandry, reproduction, larval rearing and weaning, grow-out (including nutrition), and health.

Other related projects include

- Visual development in the WA dhufish (FRDC Project 98/329)
- Intensive cultivation of a calanoid copepod for live food in fish culture (FRDC Project 96/398)
- Use of cultured copepods as food for WA dhufish (Aquaculture Development Council)
- Health problems in Western Australian dhufish (FRDC Project 98/328).
- Determination of the biological parameters required for managing the fishery of Western Australia (FRDC Project 96/103)

2.2 Potential of WA dhufish as an aquaculture species

Dhufish is a premium quality table fish, with a firm white flesh. It is highly priced compared with other local species, with a beachfront price of around $10.00 per kg for whole fish (1999). While there is a firmly established local market, there is also potential for expansion of new markets as fish with similar flesh quality are in high demand in both domestic and export markets. These factors, combined with limitations in supply indicate enormous potential for the culture of dhufish (Barnetson unpub). At the ADU, initial investigations into the possibility of supplying juvenile dhufish to the aquarium trade has also been explored, with a small number of dhufish juveniles being sold for $50.00 per fish. These initial sales were well received and prompted further enquiries by interested aquarists.

Considerable advances have been made in the culture of dhufish at the ADU. The number of juveniles produced has increased from 24 to 2000 in just 5 years of research (at 100 days post hatch). This represents an increase of more than 80 times. This is a reflection of improvements in both the number of fertilised eggs produced and the survival of larvae and juveniles. Satisfactory techniques have been developed for all the crucial stages in their life history. Dhufish culture, however, is still in its infancy and there are many areas that require further research, in particular egg production, larval rearing, nutrition and health. With continued improvements in these and other areas, the production of juveniles should continue to increase.

2.3 Need

The WA dhufish is a premium quality finfish with attributes suitable for aquaculture. Continued research into developing culture techniques for WA dhufish is necessary to 1) provide a high value species to the aquaculture industry in WA; 2) alleviate fishing pressure on the wild population of dhufish by meeting demand with farmed fish and enabling better management of the fishery.

1) There are currently several companies committed to farming marine finfish in WA. The species available for culture (snapper and black bream) have low to medium level prospects for price and markets. An urgent need exists for the development of technology suitable to culture a high priced, market driven species to support the endeavours of this fledgling industry. It is probable that the cost of dhufish culture would be similar to that of other marine finfish species. Therefore the high economic value of the WA dhufish would result in higher profit margins and more flexibility in the way the industry can develop and grow. This may be particularly important to establish an economically viable marine finfish farming industry in WA.

There are concerns regarding the use of seacages for the grow-out phase of marine finfish farming along the WA coastline. These concerns include the limited number of potential protected sea-cage sites along the high-energy coastline of WA and the perceived environmental impact of seacages, including damage to seagrass beds and euthrophication. These problems can be minimised by the establishment of land based facilities, as these facilities can clean up effluent water prior to discharge, and it is not necessary for them to be located in protected sites. Land based grow-out facilities are however, more expensive to establish than sea-based grow-out cages and the greater potential margins offered by a high value species will be very important. In addition it is likely that techniques developed for this deep water reef dwelling fish will be transferable to other highly valued species occupying a similar niche.

2) Fisheries WA have recently identified dhufish as a species "at risk" and requiring close monitoring. The development of reliable culture technology for dhufish and the subsequent establishment of a dhufish culture industry has potential to alleviate commercial fishing pressure, by enabling demand to be met by farmed dhufish and potentially enabling restocking to be undertaken.

Little is known about the biology and ecology of dhufish in the wild. The lack of information available has meant that Fisheries WA management strategies (bag and size limits) have been developed in the absence of rigorous data (Sudmeyer *et al*, 1990). More information about the life history of dhufish is clearly important. Information gained during the culture project has already been used by fisheries researchers and managers. For example, prior to the successful culturing of dhufish larvae at the ADU, dhufish larvae had never before been observed. In addition, research conducted as an adjunct to this project (funded by the Recreational Fishing Advisory Committee) indicated that dhufish caught in the depths of over 20 metres are unlikely to survive due to barotrauma related injuries. If substantiated with further research, this has major implications for fisheries management in that current practice is to impose a size limit and return undersized fish to the sea. Therefore the research from this prospect will have broader ramifications than just the aquaculture industry.

2.4 Benefits

- The Western Australian marine finfish farming industry will directly benefit from the development of culture technology for the WA Dhufish.

- The management of the wild WA Dhufish fishery will benefit from this research.

- The recreational fishing community (and the general public) will benefit due to the flow on effects of better fisheries management strategies and reduced commercial fishing pressure on dhufish stocks.

2.5 Objectives

Part A. Project No. 96/308

A1.　Reliable production of fertilised eggs (in excess of 100,000 eggs on five occasions during 1998/9

A2.　Successful production of larvae (minimum of 500,000)

A3.　Successful production of fingerlings (minimum of 10,000)

A4.　Development and documentation of production procedures for commercial production of fingerlings

A5.　Development of pelletised grow-out diet

A6.　Production of market size fish in one year

A7.　Increased knowledge of the species

Part B. Project No. 1999/322

B1.　To increase knowledge of the species, in particular reproduction, larval rearing and weaning

B2.　To improve techniques for hormonal induction of ovulation

B3.　To achieve in-tank spawning of F1 fish

B4.　To achieve out-of-season spawning of F1 fish

B5.　To assess capacity of cryopreserved sperm to fertilise eggs

B6.　To reliably produce fertilised eggs

B7.　To refine larval rearing techniques

B8.　To successfully wean larvae

B9.　To develop a suitable grow-out diet

B10.　To produce F2 fish

B11.　To produce a hatchery manual on dhufish culture

Objectives listed under Part B are for a three-year project, of which only one year was completed. Although these objectives (with the exception of B3, B4, B10) were addressed to some degree, the thoroughness of their treatment was limited by the short life-span of the project.

Chapter 3: Biology of the dhufish

Glaucosoma hebraicum or West Australian dhufish belong to the family Glaucosomatidae. Their distribution is limited to the south-west coast of Australia, from the Recherche Archipelago in the south to Shark Bay in the North (Hutchins and Swainston, 1986; McKay, 1997).

Little is known of the biology and ecology of the dhufish. Some studies have been conducted on adult dhufish in the wild, however, the early life stages (larvae and juveniles) have received little attention. In fact, until dhufish larvae were first successfully reared at the ADU, they had never been identified in the wild. Consequently the location of nursery grounds is unknown, as is the natural diet, and growth rates of larvae and juveniles.
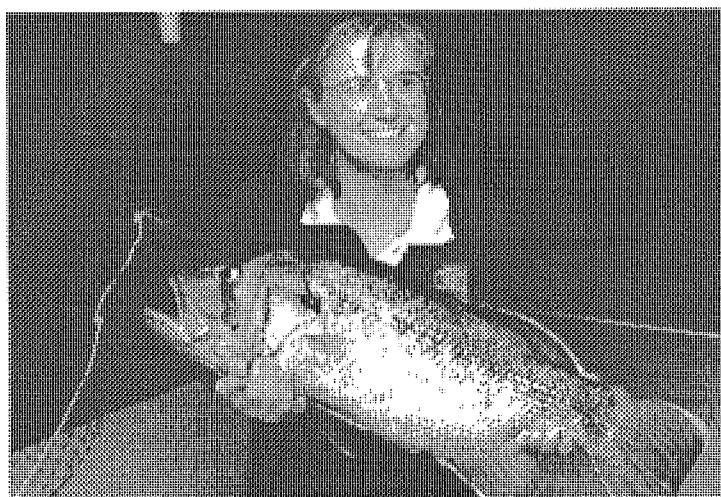
Figure 1: **Dhufish from the captive breeding program at the ADU.**

In the wild, adult dhufish grow to a maximum total length of 1200 mm and a maximum weight of 26 kg (Hutchins and Swainston, 1986). Adult dhufish feed predominantly on reef dwelling fish, including wrasse, leatherjacket, eels and mullet. Crustaceans (rock lobster, prawns, crabs) and molluscs (octopus, squid, cuttlefish) also contribute to the diet (reviewed in McKay, 1997). They can be found in waters up to 200m in depth, close to submerged reefs or rough bottom and frequent caves and overhanging ledges (McKay, 1997).

Dhufish are multiple spawners (ie they spawn more than once during the spawning season). In the wild, they spawn between December and April, with a peak in spawning activity in late January or early February (Hesp and Potter, 2000). As the spawning season approaches, heralded by increased water temperature and day-length, the ovary and testes increase in size. The weight of the gonad as a proportion of its body weight is called the gonadosomatic

index (GSI), and is used to determine the spawning season for fish in the wild. The GSI for female dhufish increases from 0.8 in July to 2.8 in January (Fig 2). In males the changes are less dramatic; the GSI increases from 0.08 in July to 0.20 in January. In the wild, the majority of female and male dhufish first reach sexual maturity at around 250-299 mm and 250 to 400 mm in length respectively (Hesp and Potter, 2000). Male dhufish can be distinguished from female dhufish, once more than approximately 2kg in weight. In male dhufish the 4th soft dorsal ray on the dorsal fin is extended into a white filament (McKay, 1997). In the female, the 4th ray does not protrude, but is flush with the other rays.
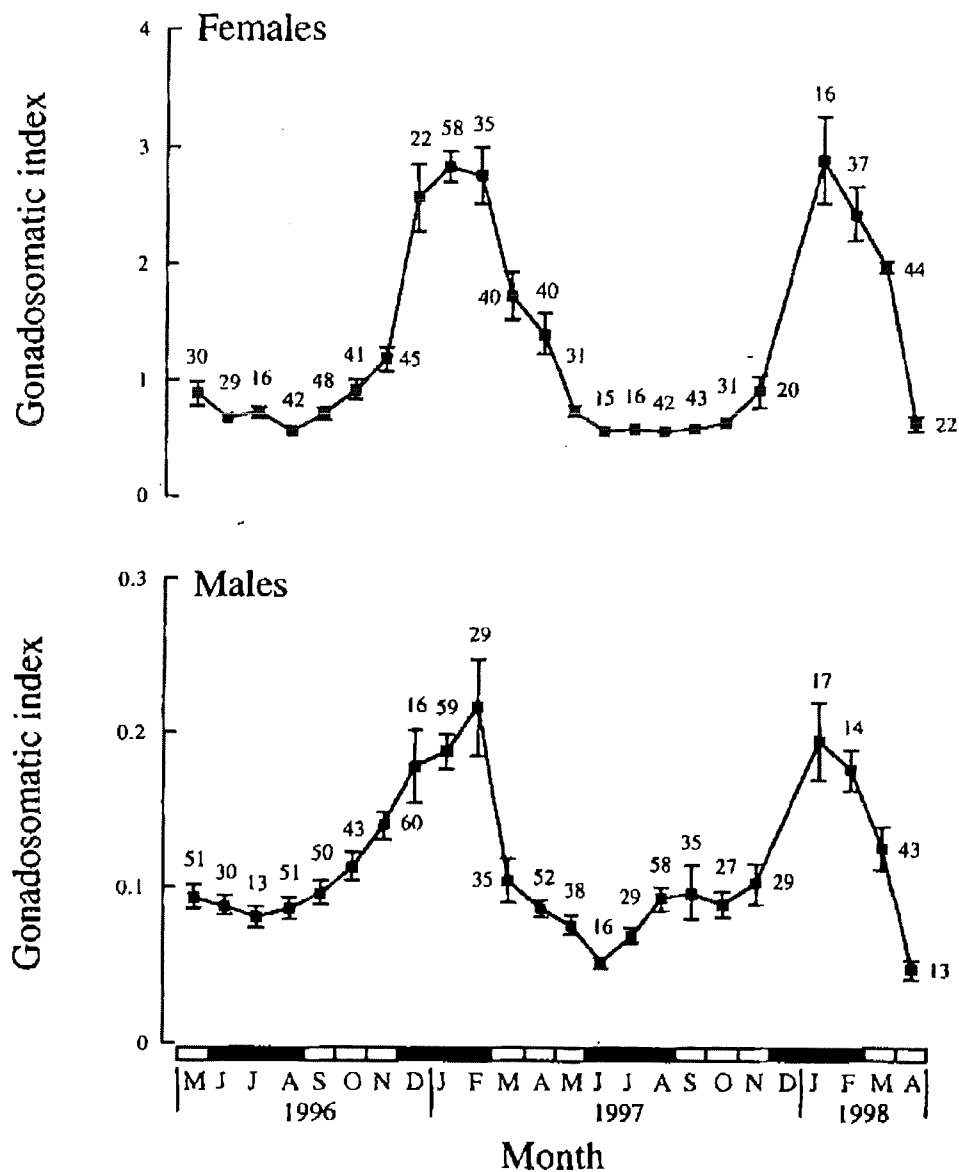
Figure 2: Mean monthly gonadosomatic indices +1SE for female and male dhufish caught in offshore waters between Geraldton and Perth between May 1996 and April 1998. Numbers of fish in each month are shown above each mean. (from Hesp and Potter, 2000).

Chapter 4: Broodstock

4.1 Collection

The preferred method for broodstock capture is by handline. Droplines and traps have also been trialed. Although droplines can be a highly effective means of catching dhufish, the stress of capture is higher than by a simple handline, due to the increased length of time the fish may be on the hook before landing. Trials using traps were conducted in cooperation with Fisheries WA. These however proved unsuccessful partly because dhufish seldom enter a trap, but also because trap-captured dhufish sustained a high degree of damage rendering them unsuitable as broodstock. The degree of damage was greater than that sustained by other species in the same trap.

Once captured, the fish should be landed directly into a tub filled with seawater. A 250 L insulated plastic tank, with a well-secured lid, is suitable for holding up to five 2 to 5kg dhufish. Once the fish is in the tub, the hook can be gently removed using long nosed pliers. If the hook is difficult to remove (ie in the gill rakers or gut), the line should be cut. The fish will normally release the hook within a few weeks of capture. Surgical gloves or other smooth gloves should always be worn when handling dhufish, in order to prevent loss of scales and damage to the mucous layer.

All bony fish have one of two types of swimbladders. Some species, known as physostomes have a pneumatic duct connecting the swimbladder to the gut. When a fish changes depth, the air in the swim bladder expands due to the reduced external pressure. During pressure change, physostomes reduce pressure by expelling gas into the gut via the pneumatic duct. Other fish, including dhufish are physoclists and lack a pneumatic duct. Their swim bladder is enclosed, but is serviced by a capillary bed, which allows gas to move to and from the swimbladder. When pressure change is gradual (eg when the fish voluntarily moves to shallow water), excess gas can be removed through the blood stream. During rapid pressure change (eg during capture by line), however, the excess gas cannot be cleared rapidly enough, resulting in supersaturation of the blood (leading to decompression sickness) and rupture of the swimbladder due to excess pressure (Appendix A).

The swimbladder of the dhufish, therefore, must be vented immediately after capture. Swim bladder deflation is performed by inserting a large bore (16 or 18 gauge) sterile hypodermic needle through the body wall into the swimbladder. The simplest and most effective place to insert the needle is 5 to 7 scales toward the tail from the tip of the operculum (just below the lateral line; Fig 3) until air bubbles out and the pressure in the swim bladder is dissipated.

A study, funded by the Recreational Fishing Advisory Committee and conducted by the ADU, found that dhufish caught from depths of greater than 20m sustain a higher incidence of liver, heart and swim bladder haemorrhage, exophthalmia, and swim bladder rupture compared with fish caught from shallower waters (Appendix A). Severely affected fish normally die within hours of capture. Dhufish broodstock are caught in shallow water to prevent this occurrence. A study has recently commenced by Fisheries WA to investigate the effects of capture depth on long term survival. However, at the ADU capture is limited to depths of less than 25m to maximise survival. At the ADU, most dhufish broodstock are caught during the winter months as they are generally easier to catch in shallow waters at this time. During the spawning season (Summer/Autumn) the majority of dhufish appear to migrate to deeper waters, resulting in additional time and therefore cost to capture them from shallow waters. If sexually mature dhufish are being sought, the best time to catch these fish is early in the spawning season (ie mid-December to mid January in Fremantle).
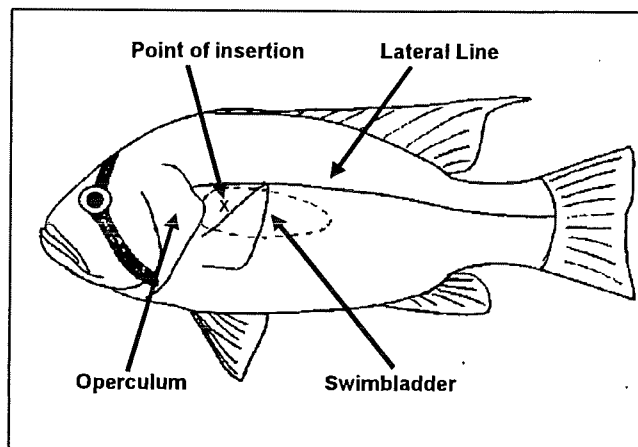
Figure 3: Diagram showing location of and point of insertion for deflation of the swimbladder

While at sea, the fish are transported in the 250 L tub described above. A well-secured lid must be used to prevent the fish jumping out, and to help retain water in the tub. When the boat is under-way, it is essential that the tub be kept completely full of water. This reduces water movement and so prevents the fish from hitting the sides of the tank by wave motion. Water should be exchanged regularly, either by bucket, or a submersible pump. A constant supply of oxygen should also be applied.

The fish can be transferred from the boat to road transport in a suitably sized covered tub, containing water. Alternatively a smooth vinyl sling, closed at both ends may also be used over short distances. Smooth plastic gloves are always worn, and care taken, as fish are normally stressed and flighty at this time. Road transport to the hatchery facility should be in a suitably sized tank (250L or greater depending on the size and number of fish and the distance to be transported) with a well secured lid. A generator may be used to run a blower to aerate the water. Other options are to use oxygen from bottles, or a battery-operated

aerator. If the fish are to be transported long distances, it may be necessary to change the water during the journey to remove toxic waste products and maintain a suitable temperature. This can be done using a submersible pump to pump directly from the ocean. Care should be taken to ensure that the incoming water is clean and free of sediment. Using these methods, survival of broodstock to the hatchery is greater than 95 %.

4.2 Quarantine

Fish caught from the wild are normally heavily infested with a wide range of ectoparasites, including isopods, copepods and gill fluke (Pironet and Jones, 2000; Appendix P). For this reason fish are placed in 4000L quarantine tanks, when they first arrive at the hatchery. The tanks are fitted with well-secured nets to prevent jumping. The fish appear calmer at low light levels so black plastic is used to cover up to 90% of the tank surface to keep the fish in the dark. This preference for low light conditions immediately after capture is likely due to the low light conditions in the fish's natural habitat; ie rocky reefs and caves (McKay 1997). The quarantine tanks are located in an area that is remote from other stocks of dhufish. Two freshwater baths 2 weeks apart are then applied to eradicate the parasites and prevent infection of established broodstock (see Chapter 8). After the period of quarantine the fish are anaesthetised, weighed and measured (see later this chapter). They are then implanted with a Passive Internal Transponder (PIT) tag (Central Animal Records, Keysborough, Victoria). These can be read with a Trovan® scanner and so enable individual identification of each fish. The tag is implanted intraperitoneally using a commercial applicator (Fig 4).
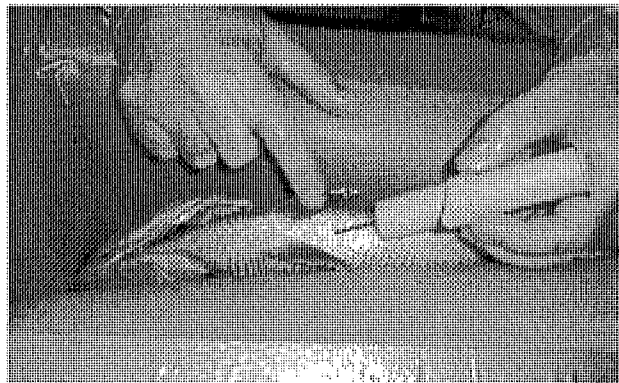
Figure 4: **Dhufish broodstock being implanted with a PIT tag using a commercial applicator**

Fish are then transported to the appropriate broodstock tank.

4.3 Tank design

At the ADU dhufish broodstock are held in cylindro-conical tanks ranging in size from 25,000 to 40,000 L. Smaller tanks have been trialed, but were unsatisfactory, as the larger fish did not feed and the females did not sexually mature (F.Pironet-Bosc, Ecloserie d'Ambararata, Aquamen, Moroondava, Madagascar, pers.com. 1999). There are several reasons for

choosing a cylindro-conical tank design for finfish broodstock. Firstly, uneaten food and faeces accumulate in the central bottom region where they can be easily removed by periodically opening the bottom drain. The tank hydrodynamics also promote efficient mixing of the water and hence the maintenance of homogenous conditions. Further, the design ensures that any fertilised eggs in the surface waters of the tank are delivered to the egg
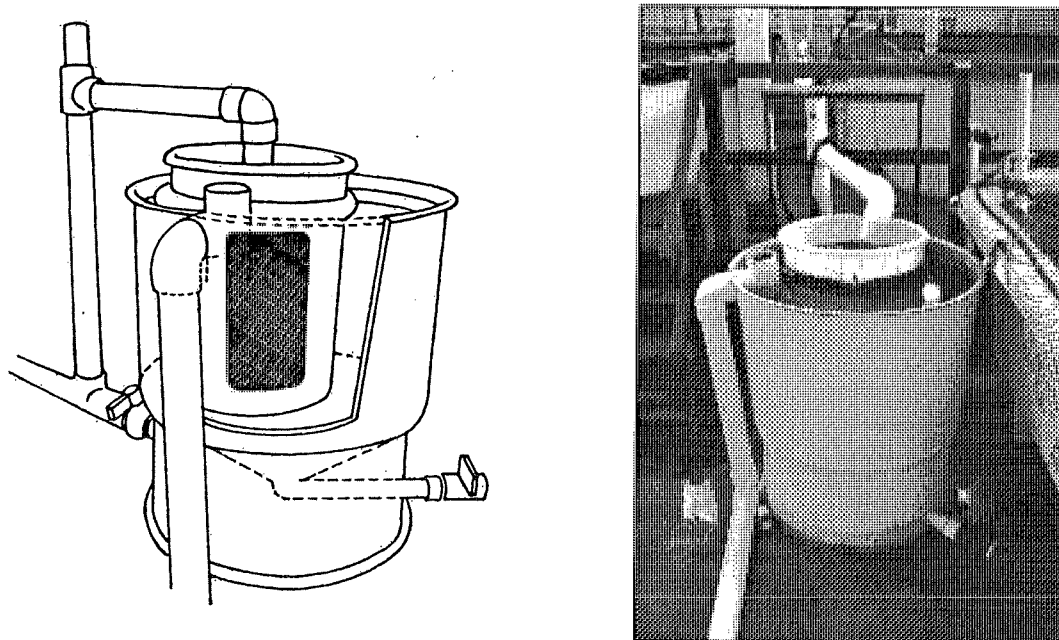
Figure 5:   **Floating egg collector**

collector in the outflow.

Although in-tank spawning is very rare in acclimated dhufish (only two fish have spawned in 5 years of research), each tank is fitted with an egg collector as a precaution. Dhufish eggs are positively buoyant. The water level in the broodstock tank is set by an overflow opening, or pipe, which directs the overflow water to an egg collector (Fig 5). The eggs are thus skimmed from the surface of the tank and collected in an egg net, set within the egg collection vessel, as the water is discharged. A water flow rate equivalent to approximately eight tank volumes per day is provided to the broodstock tanks to ensure that all of the eggs are collected within 12 hours of spawning.

4.4 Husbandry

Dhufish are relatively sedentary fish, however, they are prone to being startled by loud noises, rapid movements and sudden changes in light intensity. Therefore all tanks must be fitted with a well-secured net, to prevent the fish from jumping out. In addition, all tanks containing dhufish broodstock at the ADU are partially covered with black plastic to provide a shaded area. The dhufish tend to prefer the shade, only coming out in the open for feeding.

The maximum stocking density required for holding dhufish broodstock, while still allowing reproductive development has not been investigated. At the ADU, stocking density is kept low, at less than 1kg of fish per 1000L. It has been found that only 1 male per tank (and usually the largest one) sexually matures (Appendix J). For this reason only two males are placed in each broodstock tank with one being substantially larger than the other. Although only the largest male would be expected to mature, if the larger male is in poor condition and so fails to mature, the smaller male is likely to mature in its place. Five or six females are placed in each tank. Fish up to 8kg have been held successfully, however, at the ADU fish between 3 and 6 kg are preferred. This is a compromise between the egg production potential (larger fish usually produce more eggs) and ease of handling.

At the ADU there are two sources of salt-water: salt-water bore and ocean water. Ocean water is pumped from the ocean, while bore salt-water is pumped from 18m below the ground approximately 10m from the shoreline. Ocean water is currently the preferred water source as initial trials of holding wild-caught dhufish in bore-water have been unsuccessful, with many fish dying after 6 months of exposure to the bore-water (Appendix B). Interestingly, some hatchery-reared dhufish have been held on bore-water for more than a year and remain healthy. Bore water at the ADU has high carbon dioxide levels and low pH. These, or a combination of these features are thought to be the cause of the problems experienced with holding wild-caught dhufish in borewater at the ADU. Bore-water however has several advantages over ocean water as it is not influenced by weather and tidal conditions, has a more stable temperature and is easier to maintain due to reduce filtration requirements. Trials are therefore continuing to see if the bore-water can be made more suitable for rearing dhufish by degassing.

In the meantime, each broodstock tank is supplied with air and ocean water filtered to 10μm (nominal). Both recirculation and flow through systems are currently in use for dhufish broodstock. Water temperature ranges annually between 15 and 26°C. Optimal temperatures have not been determined, however, it is likely that temperature between 16 and 23°C is most suitable. These temperatures are similar to those experienced in the wild (Pearce, 1991) and the incidence of exophthalmia appears to increase at temperatures above 23 °C (Dr F. Stephens, School of Veterinary Studies, Murdoch University, pers. com, 2000). pH of ocean water is normally between 8.1 and 8.2 with salinity ranging between 33 and 35 ppt.

Tanks are vacuumed regularly to prevent build up of waste products on the tank bottom. The drain valve is also regularly purged. Uneaten food is removed by dip-net soon after feeding.

Dhufish are very sensitive to poor water quality and have developed keratitis on three occasions following: their exposure to short periods of stagnant water (on two occasions); and after decayed food was removed from a tank (one occasion; F.Pironet-Bosc, Ecloserie d'Ambararata, Aquamen, Moroondava, Madagascar, pers.com. 1999). Following exposure to

the poor water quality, the fish immediately ceased feeding and the lens of their eyes turned cloudy. On one of these occasions all the fish in the tank died within 10 days of the occurrence. This emphasises the need to be vigilant in maintaining tank hygiene.

4.5 Feeding and nutrition

Feeding provides a good opportunity for assessing the health of broodstock, as decreased appetite is often an indicator of poor health. For this reason, an accurate record of fish behaviour, feed intake and composition should be maintained. In broodstock tanks, which typically have a small number of fish in them (less than 10), it is possible to identify each fish based on size and external markings. Close observation during feeding can therefore yield information on the health of individual fish.

Little is currently known about the nutritional requirements of dhufish broodstock. At the ADU, dhufish broodstock are fed a rotating diet of freshly-thawed frozen garfish, mullet, pilchards, squid, whiting, prawns and octopus. In the wild, wrasse comprises the majority of the diet (reviewed in McKay, 1997), however, a reliable source of wrasse has not been identified and is not currently a major component of the captive broodstock diet. Once thawed, the food may be chopped to an appropriate size, however, dhufish have a large mouth and can consume food that is large relative to their body size. A fish of 1kg or greater can consume a 15 cm pilchard. Dhufish broodstock are discerning feeders, and will ignore poor quality seafood. Wild-caught broodstock have accepted moist pellets (Appendix D), however, these are not used regularly at the ADU. Hatchery-reared broodstock readily accept moist pellets and these are fed regularly to supplement the flesh diet.

The diet for both wild-caught and hatchery-reared broodstock is supplemented regularly with vitamins and minerals (Appendix C). In addition wild-caught broodstock are also treated with the glucan immunostimulant Macrogard® (Vetafarm). For wild-caught broodstock, vitamins/minerals and Macrogard® are administered together in a gelatine capsule in a ratio of 6 to 1. The capsule is inserted into the feed prior to feeding. Approximately 0.6 g of vitamins/minerals and 0.1g Macrogard® are given per kilogram of fish at each feed. Capsules are administered 3 times a week for two weeks every month. For hatchery-reared broodstock, the vitamins are incorporated in a moist pellet (at an inclusion rate of 0.5%/kg) and no Macrogard® is given. The vitamin-enriched pellet comprises about 20% of the total diet (see Appendix D for recipe for the moist pellet).

4.6 Handling

Crowding

When dhufish require handling or a treatment such as a fresh water bath, the fish are herded into a crowd. The crowd is constructed of a buoyant hoop (1.3m in diameter) with a thick cylindrical plastic "bag" constructed of black builders plastic (300mm deep) attached around the edge of the hoop. The fish are then slowly herded into the crowd by sinking the leading edge of the crowd to allow the fish to swim in. In the large broodstock tanks the water level is lowered to knee depth and 3 or 4 people are used to quietly herd the fish into the crowd (Fig 6). While the water level is low, the fish are generally quiet and compliant, as long as sudden movements do not startle them. Once the fish are in the crowd, it is covered with a clear plastic cover. The cover is secured with strong clips to prevent the fish jumping out during anaesthesia or other treatment. Both black and white crowds have been trialed at the ADU. The fish generally appear more settled in black crowds, however, in tanks which are all white (rather than the usual dark walls and white floor) a white crowd if generally better, as the fish will not enter into a black one. Crowds of 300 L and 450 L have been used successfully and are recommended. Up to ten 2kg fish or three 6kg fish are held in each crowd.

Figure 6: **Crowding dhufish broodstock using circular floating crowd.**

Anaesthesia

Broodstock require occasional handling to check gonadal condition or to administer medical or hormone treatments. For all such procedures anaesthesia is recommended. Dhufish are generally anaesthetised in a floating crowd. Occasionally the anaesthetic is added directly to the tank, however this is expensive and is normally only done if the volume of water can be

reduced to less than 1500L (to reduce the amount of anaesthetic required. During anaesthesia oxygen is always supplied.

Two anaesthetics are regularly used to anaesthetise dhufish broodstock: AQUI-S (AQUI-S New Zealand Ltd) and 2-phenoxyethanol (Sigma cat. no. P1126).

- AQUI-S is derived from clove oil and is reported to be non-toxic to humans, although gloves are always worn as a precaution. At 0.02 ml.L^{-1}, AQUI-S is used for procedures which require light sedation, such as administering injections, taking blood samples, weighing, measuring, transporting and conducting ovarian biopsies. AQUI-S at 0.07 ml.L^{-1} is used for heavier sedation. To date AQUI-S has not been found to be suitable for full surgical anaesthesia in dhufish. AQUI-S is dissolved in fresh water before use. Oxygenation must be kept to a minimum to prevent the anaesthetic foaming on the surface. The solution should be added slowly to ensure it mixes thoroughly in the crowd. The action of AQUI-S is very gradual and so the anaesthetic is added slowly and the reaction of the fish observed carefully to prevent over-dose.

- 2-phenoxyethanol (0.3ppt) can induce full surgical anaesthesia. It is reported to be highly toxic and so full precautions must be taken when handling the chemical (eg gloves and eyewear). Due to its toxicity, the use of this anaesthetic is now limited to occasions where full surgical anaesthesia is required such as stripping. 2-phenoxyethanol must be completely dissolved before adding to the crowd. Undissolved 2-phenoxyethanol appears to irritate the fish and causes violent thrashing. Therefore, the required volume of the anaesthetic is added to a 2L bottle containing fresh water. The bottle is shaken to aid mixing, and then left to stand. The undissolved anaesthetic will settle to the bottom and the dissolved portion can then be decanted into the crowd. This process is repeated until sufficient dissolved anaesthetic has been administered. Although not as gradual as AQUI-S, the action of 2-phenoxyethanol is still quite slow and so is also made gradually

Once the required level of anaesthesia is reached, the fish can be removed from the water and various procedures performed. Following the procedure the fish are revived. To revive the fish it is placed in anaesthetic-free water. Using smooth gloves, the fish should be held by the lower jaw and the caudal peduncle and moved gently backward and forward. This forces water over the gills. Alternatively the gills of the fish can be held over an air-stone bubbling air or oxygen. Fish recovering from 2-phenoxyethanol-induced anaesthesia tend to recover suddenly and may dart out of the reviver's hand. This may damage the fish if it strikes the wall of the tank. Fish recovering AQUI-S-induced anaesthesia recover very slowly and may take 5 minutes before they regain buoyancy. During this time they tend to swim on their side on the bottom of the tank. This may damage the eyes or cause other abrasions if the tank

surface is not completely smooth. Therefore, once the fish are ventilating, they are normally released into a floating crowd (described above) until full buoyancy is regained.

Handling procedures

Once anaesthetised, the fish can be removed from the water and various procedures performed. Gloves are always worn when handling dhufish to prevent mucous loss. Dhufish have large, protruding eyes, which are prone to damage during handling. Problems include superficial damage to the cornea and haemorrhage and swelling of the eyeball (see section 8.3.2). For this reason, a number of systems have been designed for working specifically with dhufish in order to prevent eye damage.

- Following anaesthesia the fish are normally transported in a sling, closed at one end. Smaller fish may be moved, without anaesthesia, in a covered tub containing water, large enough to hold them comfortably. This is sometimes easier as it avoids the need for anaesthetics, but is not recommended for fish heavier than 2kg.

- The fish can be placed on a flat, but soft surface, such as a block of high-density foam covered in plastic. A section of the foam is cut away, to create a hollow for the eye to rest in, thereby eliminating contact of the eye with any surface. This is suitable for some minor procedures, and also those that require access to the whole fish.
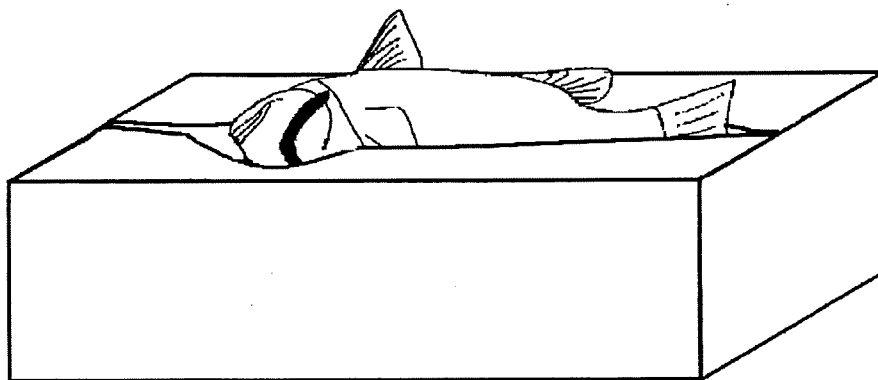
Figure 7: Dhufish in a foam restraint.

- A foam restraint is useful for procedures that require access to the ventral surface only such as blood sampling, implanting hormone pellets and pit tags, administering intramuscular and intra-peritoneal injection, and conducting ovarian biopsies (Fig 7; adapted from N.W. Pankhurst, School of Aquaculture, University of Tasmania, pers.com. 1994).

The restraint is a large, deep block of high-density foam (at least 1 ½ x the dimensions of the fish) with a slit cut length-wise into the centre. This slit is shaped at one end to create a gap large enough to fit the head and eyes comfortably (this alleviates pressure on the eyes). The remainder of the slit should be kept narrow to ensure firm pressure along the body. The slit and block are then covered in plastic. The plastic covering should be wet prior to use to prevent damage through abrasion. An anaesthetised fish can then be inserted deeply into the plastic covered foam, belly up. The advantage of this system is that the fish is safely restrained, allowing the operator to concentrate on their task.

- For weighing and measuring, a hanging cradle is used (Fig 8). It is constructed of 6 mm PVC sheet and is designed to hang from a spring balance. A large hole is cut in the region where the eye would lie, to prevent the eye from resting on the hard plastic. Graduations on the cradle allow simultaneous measurements of length and weight.
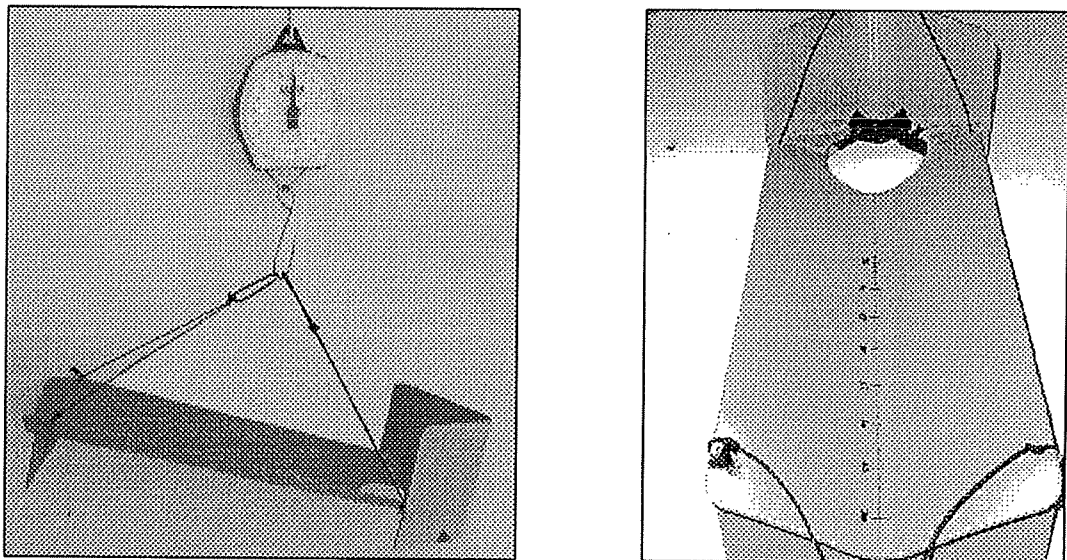
Figure 8: Cradle for weighing and measuring dhufish.

Chapter 5: Reproduction

5.1 Overview

Although some species of marine finfish can complete sexual maturation in captivity, for many species, normal maturation through to spawning does not occur under culture conditions. This is usually due to stress imposed by the culture environment (reviewed in Pankhurst, 1998). In some cases, the species may acclimate given sufficient time (perhaps several years), however, as is the case with snapper *Pagrus auratus,* a truly domesticated stock (ie grown in captivity from eggs) needs to be produced before the species will spontaneously complete sexual maturation and spawn in captivity. Wild-caught acclimated snapper do not normally ovulate or spawn naturally in captivity (Cleary, 1997). Hatchery-reared snapper do however spawn readily in tanks. At the ADU, hatchery-reared snapper spawn unaided in their tanks on a daily, year round basis. In the absence of spawning broodstock (either acclimated or domesticated) hormonal intervention is required to artificially stimulate the fish to complete maturation.

Female dhufish caught from the wild and acclimated to the culture environment do not naturally complete maturation; they usually fail to ovulate (release of eggs from ovarian tissue into the oviduct) and consequently fail to spawn (release of eggs from oviduct into water column following courtship behaviour). In this regard, dhufish are similar to snapper. Currently at the ADU there are 95 hatchery-reared dhufish approaching first maturity. It is hoped that, like snapper, these fish will spawn naturally in their tanks. It was hoped that this would occur in January 2000, however, it now appears that this group of fish may be all females. If this is the case, the lack of males would have inhibited spawning. At least some of the females sexually matured in that season. Acclimated males (caught from the wild) have now been stocked with the hatchery-reared fish and spawning is now expected in January 2001.

In-tank spawning is the preferred means of collecting eggs, as it avoids the complications of hormone treatment and stripping. In the absence of spawning hatchery-reared dhufish however, hormone therapy is required to stimulate maturation and ovulation. On two occasions (during the last 5 years of research), female dhufish have spawned in the tank following hormone treatment. The eggs were fertilised by the resident male. This, however, is rare and the fish normally need to be stripped. Trials have been conducted to try to reliably stimulate spawning using hormones. These have not been successful (Appendix F).

5.2 Hormones

In a reproductively healthy female, Gonadotropin Releasing Hormones (GnRH), produced by the hypothalamus, trigger the release of Gonadotropins (GtH) from the pituitary. The GtHs

then stimulate the production of reproductive steroids, which in turn cause oocyte maturation and ovulation (Fig 9). There are two main approaches to artificially inducing ovulation in fish: treatment with GtH or GnRH preparations. GtH preparations mimic the action of the fish's natural gonadotropin and so stimulate the production of reproductive steroids and in turn stimulate final oocyte maturation and ovulation. Human chorionic gonadotropin (hCG) and pituitary extracts are examples of this. In contrast, GnRH preparations mimic the fish's natural GnRH, and act directly on the pituitary to stimulate secretion of the fish's own GtH. GnRH preparations are either purified from a natural source (ie from the same or different species) or can be an analogue (synthetic copy) of a natural GnRH (GnRHa). Luteinising Hormone Releasing Hormone analogue (LHRHa) is a GnRHa commonly used in fish and is an analogue of a mammalian hormone. In some species, the release of GtH by the pituitary is controlled/down-regulated by naturally occurring dopamine. In these species, it may be necessary to administer GnRHa in conjunction with a dopamine antagonist (eg domperidone) to ensure the release of GtH and hence maturation. Ovaprim®, produced specifically for fish, is a commercial product containing GnRHa and domperidone.
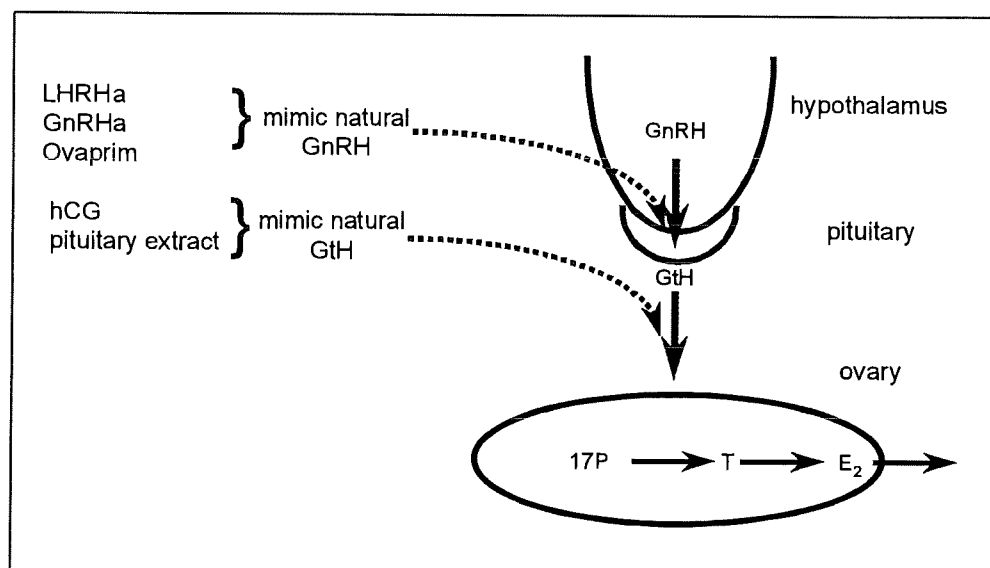
Figure 9: **The action of LHRHa and hCG on reproduction in marine fish.**

Three hormones have been trialed on dhufish: hCG, Ovaprim® and LHRHa. HCG may be purchased as Chorulon® produced by Intervet and can be purchased by prescription from veterinary suppliers. This is a commercially prepared product supplied in two vials. One vial contains hCG and the other distilled water. This product is simple to use and may be stored before dilution in the fridge or on ice until use. Alternatively hCG can be purchased from Sigma (Cat. No, C 5297) as a powder. This can then be dissolved in saline or distilled water. For ease of use, the solution can be made ahead of time, frozen in appropriately sized aliquots and thawed when required. Ovaprim® is supplied as a viscous liquid from Syndel. It may be stored in the fridge and is administered by injection as supplied. LHRHa (Des-

Gly^{10}(D-Ala^6) luteinising hormone releasing hormone ethylamide salt; Sigma L4513) is either dissolved in saline solution (as for hCG) or prepared as a pelletised implant containing 95% cholesterol and 5% copha oil (Lee *et al*, 1986; see Appendix F). Pellets and LHRHa solutions can be made in advance and stored in the freezer in appropriately sized vials. All hormones stored in the freezer should be discarded 2 days after thawing. All dissolved hormones are administered in sterile syringes with 18 – 25 gauge needles. Implants are administered using a large bore needle (1.8 mm internal diameter, 2.2 mm external diameter) with an internal shaft. At the ADU recycled needles (as supplied with microchip PIT tags) are used successfully for administering implants (Fig 4). These are stored in alcohol between use. All hormones are administered intraperitoneally (ie into the abdominal cavity). LHRHa administered in a pellet is most commonly used at the ADU with acclimated male and female dhufish (Appendix G & J). Chorulon® (hCG) is used with sexually mature females caught during the spawning season (Appendix H). There are indications that Ovaprim® is also useful in inducing ovulation, however, this has not be tested in a controlled manner and it is not currently used at the ADU on dhufish.

5.3   Egg production

5.3.1   Sources of eggs

Acclimated females

Females should be caught in shallow water (preferably <20m) from the wild during winter and spring and held in tanks as per Chapter 4. During the expected spawning season, females should be observed closely for evidence of maturity. As the developing oocytes undergo vitellogenesis, they increase in size. This results in a swelling of the ovaries and hence the abdomen. When most of the fish in a particular tank have swollen abdomens, all the sexually mature females in that tank are treated with hormones to stimulate final oocyte maturation and ovulation. To achieve this, the tank is drained as quickly as possible to reduce the period of time in which the fish are acutely stressed prior to hormone treatment. The fish are then anaesthetised, weighed, measured and an ovarian biopsy performed on females.

Ovarian biopsies are conducted to determine the stage of sexual maturity of developing oocytes, both to determine if or when hormones need to be applied and to predict the time of ovulation (and therefore when stripping is required). This is always performed under anaesthesia. A biopsy tube or cannula (a hollow surgical tube of maximum 3mm outer diameter and minimum internal diameter of 1.5mm), is gently inserted into the oviduct (the opening closest to the tail in the vent area). A commercial endometrial biopsy cannula can also be used. A sample of the gonad is then taken by either sucking the surgical tube or working the plunger of the commercial cannula as the tube is withdrawn from the oviduct. The sample secured in the cannula is then ejected into a drop of seawater on a slide and

examined under a microscope fitted with a measuring graticule. The diameter of the ten largest oocytes (developing eggs) is measured and the mean (or average) diameter calculated.

Female dhufish with a mean oocyte diameter of greater than 400μm are likely to respond to the hormone and are treated. LHRHa (administered by saline injection or slow-release pellet), hCG and Ovaprim® have been used with some success on acclimated females. An LHRHa pellet is now the hormone of choice. Doses as low as 5 μg.kg^{-1} (slow-release cholesterol pellet) have been successful in inducing ovulation, however, the results have been unreliable. LHRHa pellet at a dose of 50μg.kg^{-1} is sufficient to stimulate ovulation in the majority of fish (oocyte diameter of > 500um) within 24 to 96h of implant and is therefore recommended (Appendix G). Following hormone treatment, the females are transferred to a smaller easily accessible tank where they can be monitored closely. A 4000L tank is used for this purpose at the ADU.

Wild-caught females

Eggs may also be obtained from sexually mature females caught from the wild during the natural spawning season (Appendix H). Fish should be caught from shallow water (<30m). The best time to catch sexually mature females in shallow water is early in the spawning season (usually mid-December to mid-January). After this time the dhufish appear to migrate to deeper waters and so are difficult to catch in shallow waters.

Fish should be caught by handline. Immediately after capture the swimbladder should be deflated (see Section 4.1), the fish placed in a foam restraint (Fig. 7) and an ovarian biopsy conducted. The biopsy should be examined to determine if the fish is sexually mature. If it is not possible to have a microscope on the boat then, with practice, this can be done crudely by eye. Sexually mature females (ie those with vitellogenic, hydrating or ovulated oocytes should be injected with hCG at 1000 IU.kg^{-1}. Weight can be estimated from a measure of the length of the fish to avoid the difficult task of weighing fish at sea (Appendix J). The fish is then released into the transport tank. All of these procedures can be performed without anaesthetics if a properly constructed foam restraint is used. The use of anaesthetics at sea is not recommended due to the risk to workers of splashing onto skin and eyes. After treatment the fish are then transported to a land-based facility in an appropriately sized tank with a close fitting lid, and supplied with oxygen (Section 4.1). At the land-based facility they are placed in easily accessible tanks for close monitoring as for acclimated fish above.

Hatchery-reared females

At the ADU there are currently more than ninety 3+ year old hatchery-reared dhufish. Some of these fish reached sexual maturity in summer 1999/2000 (ie at 3 years of age), although they did not spawn. However, it is expected that these fish will spawn in the 2000/2001 season. Interestingly, no males have been positively identified amongst these fish, either through gonadal biopsy or external morphology (ie. elongated rays on dorsal fin). The absence of males (if proven) would explain the lack of spawning. Wild-caught males have now been added to the tanks. To date they have not been used in trials for hormonal induction of ovulation.

Comparison of egg sources

Although advances have been made in terms of securing a reliable supply of good quality fertilised eggs, the maximum annual egg production for dhufish has been 1.5 million. This is insufficient to support an industry. During the 1999/2000 season, success was achieved in obtaining and fertilising eggs from females caught from the wild during the spawning season, making available another source of eggs. Collection of sexually mature females in suitable physical condition is time consuming and is therefore expensive. However, this must be weighed against the cost of maintaining large numbers of captive broodstock year round: broodstock must be held in large tanks and are fed high quality food. Hatchery-reared dhufish have reached sexual maturity. If these fish spawn successfully in their tanks, without the need to administer exogenous hormones, egg production will eclipse production from all other sources. This would be particularly true if attempts to stimulate hatchery-reared dhufish to spawn out of season using photo-therm manipulation are successful. Hormonal induction of ovulation and stripping of fish caught from the wild will, however, remain important to maintain genetic diversity (particularly in early days when hatchery-reared fish come from limited numbers of broodstock), replace stock after a disease outbreak, or for advanced genetic manipulation (triploid, hybridisation). A sensible approach to egg production would be to incorporate all three sources of eggs in such a way as to take advantage of the benefits offered by each.

Monitoring egg development and ovulation

All externally fertilising species have a window of post-ovulatory viability (ie the period after ovulation in which eggs retain the ability to be fertilised) after which egg viability declines with time (reviewed in Hobby and Pankhurst, 1997). The period of peak fertility varies between species and ranges from several hours (eg snapper) to several weeks (eg trout). Reliable stripping of good quality eggs is dependent on the correct estimate of the length of this window, for the species in question. In captive female dhufish, viability was found to decrease to 50% within 3-5h of ovulation (Cleary et al, 1999; Appendix I). Therefore the fish need to be monitored closely so that eggs can be stripped and fertilised as soon as possible

after ovulation. For this reason, once females are treated with the hormone at the ADU they are placed in a 4000L tank where they can then easily be observed and lightly anaesthetised for ovarian biopsy. This simplifies the process of regular monitoring of egg development.

At the time of hormone treatment, oocyte diameters are greater than 500μm. Oocyte diameter gradually increases. During this time biopsies are conducted every 12h. After the oocytes reach 800μm, they begin to hydrate (the final stage of egg development before ovulation). In acclimated female dhufish, this will normally only occur following hormone treatment. When this stage is reached it is likely that the female will ovulate within the next 12h and the fish should be monitored closely. A fully hydrated egg is normally between 1000 and 1200μm in diameter. Ovulation occurs soon after hydration is completed. The point when the hydrated (clear and large) oocytes have separated from the ovarian tissue (and other smaller developing eggs) can be identified from a biopsy sample. Alternatively, applying light pressure to the abdomen should cause any ovulated eggs to flow freely out of the oviduct, provided the oviduct is not blocked. As a female approaches ovulation, a source of sperm must be located (Section 5.4) before the female is stripped (Section 5.5).

5.4 Sperm collection, storage and quality assessment

Male dhufish typically produce small volumes of sperm. In many other cultured species, the testis comprises 5-10% of the total body weight during the spawning season (eg black bream, snapper), and they typically produce large amounts of sperm. In contrast, the testes of the male dhufish weighs as little as 0.1% of the body weight during the spawning season (Hesp and Potter, 2000), and they typically produce less than 1 ml of sperm at a time. Therefore, securing a reliable supply of dhufish sperm is more difficult than for many other cultured species.

5.4.1 Source of sperm

When a female is approaching ovulation, a source of sperm must be located. There are potentially three main sources of dhufish sperm available: sperm stripped from live acclimated males; sperm collected from the testes of fish caught from the wild; and cryopreserved sperm. Due to the limited supply of sperm from male dhufish, all these sources of sperm need to be utilised optimally and research must continue to enable this.

Acclimated males

Stripping

Male dhufish will spermiate in captivity, however, it has been found that only one male per tank will spermiate. This is usually the largest, dominant male. A smaller male will spermiate

if the larger one is removed or is ill. For this reason, at the ADU, it is common practice for each broodstock tank to contain only two males (one larger and one smaller).

When a female is approaching ovulation an acclimated male is selected and anaesthetised. As this procedure requires the fish to spend a long period out of the water, it is important that a high degree of anaesthesia is attained. For this reason 2-phenoxyethanol is used at 0.3 ppt. Once anaesthetised, the abdominal region of the fish is rinsed with freshwater and gently towel dried to remove traces of salt water and anaesthetic. The fish is then stripped by applying pressure along the abdomen, beginning at the front of the fish (behind the pectoral fins), and moving toward the vent. A plastic apron and gloves are always worn when stripping dhufish to prevent damage to the protective mucous layer. The sperm is collected in a sterile 1-2 ml syringe as it exudes from the vent (Fig 10). When sperm is stripped it is inactive (ie cells are immobile) whereas upon contact with salt water, the sperm activates (ie becomes mobile). Once activated, sperm will be viable for only as long as it remains mobile (maximum of several minutes). To prevent activation, care must be taken to avoid contamination with seawater and the sperm should not be allowed to pool on the body surface before collection. The syringe containing the collected sperm is capped after collection and inactive sperm can be stored on ice for long periods of time (up to several days).
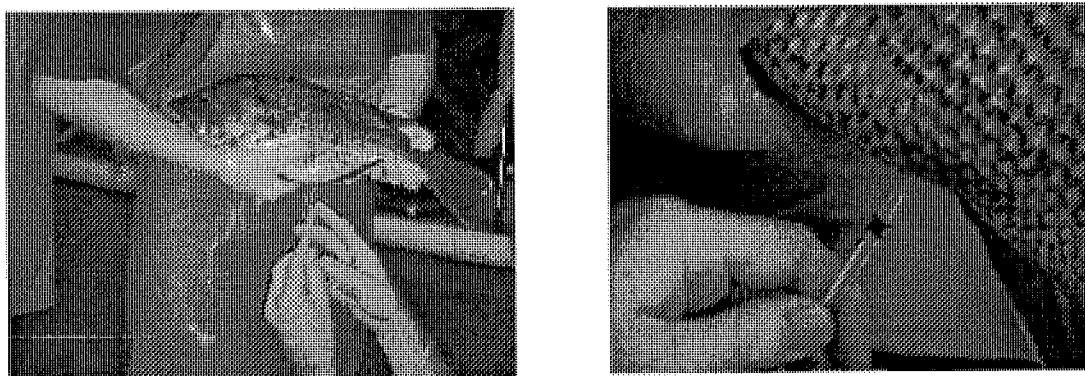
Figure 10: Stripping male dhufish

Sperm quality

The quality of the sperm is assessed before use by placing a minute drop of sperm onto a glass microscope slide with a cover slip and viewing with a compound microscope at 40x magnification. Inactive sperm cells appear as immobile dots, while active cells swim rapidly across the field of view. Any sperm cells vibrating on the spot, but not swimming are considered to be inactive. An approximation of the number of active cells as a percentage of the total number of cells in the field of view is made. This is repeated at different positions on the slide and the average of these measures gives the activity of that sperm sample. If care has been taken to avoid contamination with seawater, all sperm should be inactive (ie immobile). If the sperm has been activated, that sperm sample is discarded. Slides and

cover slips must be very clean, as residual salt is sufficient to activate sperm and may result in sperm being incorrectly discarded.

If the sperm is inactive then a single drop of seawater is placed along the edge of the cover slip. The water will be drawn under the cover slip by capillary action and will contact the sperm. Immediately after the salt water is added, activity is checked again as above. The higher the percentage of active cells the better the quality of the sperm. Sperm that is less than 70% active is normally discarded, if better quality sperm is available.

Storage

Once collected, inactive sperm can be stored on ice in the collection syringe. The quality of the inactive sperm will deteriorate with time (normally over several days, but sometimes over several hours). To ensure sperm is of the highest available quality, sperm should be collected as close to the time of ovulation as possible. Optimally, two males should be stripped to maximise the chance of successful fertilisation and hatch.

Ensuring supply

Although male dhufish readily spermiate in captivity, the small number of spermiating fish per tank and the small sperm volume produced per fish necessitates that each spermiating male be stripped regularly during the spawning season. Repeated handling and stripping over a period of 1 week reduces the volume of sperm available, as well as the number of fish spermiating (Appendix J). Treating the fish with an LHRHa implant ($10\mu g.kg^{-1}$) can overcome the negative effects of the stress of handling and stripping, by maintaining sperm production (Cleary et al, 1999). Therefore an implant of LHRHa ($10\mu g.kg^{-1}$) is regularly used at the ADU to maintain sperm supplies during the spawning season. Problems however, have been experienced in storing sperm stimulated by LHRHa implants. Sperm that isn't used immediately after stripping of LHRHa implanted fish appears to rapidly decline in quality (as determined by activity). This aspect needs to be investigated further.

Testes from wild-caught males

Due to the limitations in sperm production of acclimated dhufish, it is necessary to supplement the hatchery sperm supply with sperm from wild-caught males. In addition to stripping acclimated males, sperm has been collected from freshly killed dhufish caught from the wild by professional or recreational fishermen (Appendix K). Testes were collected from sexually mature males from the fish markets, fishing competitions, and directly from individual recreational fishermen. The quality of the sperm (determined by activity) appears to be dependant on the length of time the fish have been dead, how well the fish has been chilled after death (the colder the better, but not frozen), and the length of time the testes have been

removed from the fish. Dhufish at the fish markets are usually well chilled, however, have usually been dead for more than 72h. Average sperm activity from the fish that were sampled was as low as 27% and unsuitable for use in fertilising eggs. Average activity of sperm from fish caught at fishing competitions was 64% which is adequate (although not optimal) for fertilising eggs. These fish were killed up to 10 hours before the testes were removed from the fish. Due to logistical constraints, once removed from the fish, the testes were stored in airtight plastic bags on ice for approximately 5 hours before the activity was measured. There is anecdotal evidence to suggest that testes stored within the abdominal cavity of the fish (well chilled) will retain higher sperm activity for longer, compared with testes stored in plastic bags. Although this has not been tested in a controlled manner, the current recommended means of short-term storage of testes is to leave them in the fish and to chill the fish well. The efficacy of stripping freshly-caught wild males has not been explored.

Cryopreservation

Due to the limited supply of sperm supply from male dhufish, work has commenced to develop techniques for cryopreserving sperm. Cryopreservation offers potential for storing sperm for long periods of time. There are a number of different techniques developed for various species. The success of cryopreservation varies among species and among techniques. Therefore techniques must be optimised for each species.

Early trials have been successful in freezing and thawing dhufish sperm at the ADU and the thawed sperm has been used to fertilise eggs. Fertilisation and subsequent hatch however, have been low compared with eggs fertilised with fresh sperm.

The best technique developed so far at the ADU (see Appendix L) involves gradual dilution of dhufish sperm with a diluent containing DMSO (6.25%) and glycerol (5%) in a teleost ringers base. A known volume of sperm is added to a sterile 1.2 ml cryogenic vial. Diluent is added in four equal portions, each portion being equivalent to the volume of the sperm being preserved. The rate of dilution must be very slow to avoid osmotic shock, and a delay of at least 30 seconds is observed between successive additions. The vial containing the sperm and diluent is gently mixed during this time. These procedures should be carried out on ice, and all equipment used must be sterile before use to avoid contaminating the sample. Once the dilution is completed, the vial is capped, labelled and attached to a storage cane. The sample is then suspended in liquid nitrogen vapour just above the level of the liquid nitrogen for 5 minutes. The vial is then plunged into the liquid nitrogen. Frozen sperm is then stored in the liquid nitrogen dewar until required. Thawing is conducted in a warm water bath (30 to 40°C) for 1.5 minutes.

5.5 Stripping and fertilisation

Once sperm is collected, monitoring of the female is continued, by taking regular ovarian biopsies until ovulation is detected. The female is then anaesthetised deeply using 2-phenoxyethanol (0.3 ppt) and prepared for stripping as for males. The eggs are collected in a clean and dry measuring cylinder with a large funnel (Fig 11). When eggs contact seawater they harden, rendering them impermeable to sperm. For this reason, the abdominal region must be rinsed well with fresh water and gently dried prior to stripping.
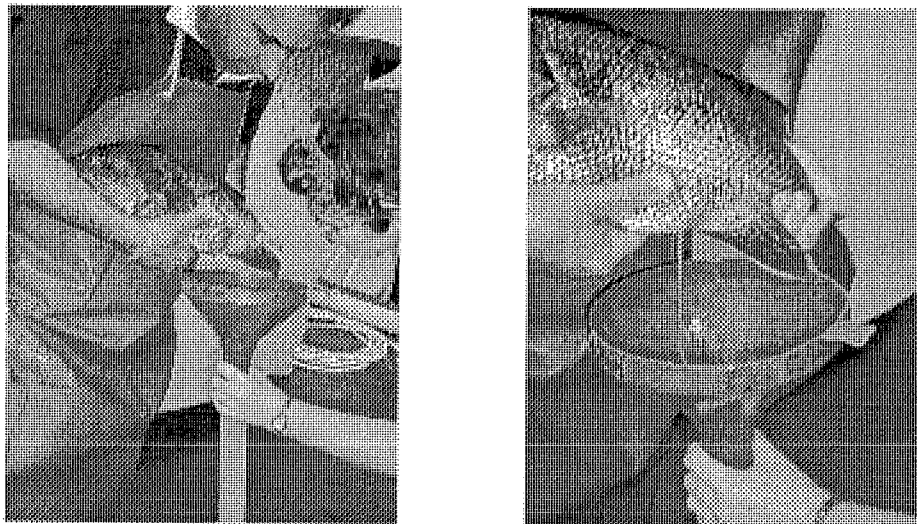
Figure 11:   Stripping female dhufish

The total number of buoyant eggs stripped from individual acclimated females at the ADU, following treatment with an LHRHa pellet at 50µg.kg^{-1}ranged from 24,000 to 300,000 over 3 consecutive strippings, 24h apart (Appendix G). A female dhufish held in a 3,000,000L public aquarium (Underwater World, Sorrento, WA) produced a total of 700,000 eggs over 5 consecutive nights following hormone treatment with LHRHa pellet at 50µg.kg^{-1}. The total number of eggs obtained from individual females caught from the wild during the spawning season and treated with hCG (1000IU kg^{-1}) ranged between 63,000 and 105,000 over four consecutive nights (Appendix H). Once ovulated, the eggs are transferred to a clean and dry 1 L glass beaker for fertilisation. The eggs are very fragile at this time and so the transfer should be conducted gently. Sperm or macerated testes is added to the eggs and gently mixed to ensure the sperm is well dispersed amongst the eggs. Seawater is added to the eggs (volume equivalent to six times the volume of the eggs) with further gentle mixing for several minutes. The suspension is then left to stand for a further 10 minutes before being transferred to the counting cone.

5.6 Rinsing and counting eggs

All eggs produced, whether by spawning or stripping, are transferred to a counting cone for counting and rinsing. The ADU counting cone is a 100L cylindro-conical polypropylene cone with volumetric graduations on the vertical walls (Fig 12). A gentle flow of seawater ($\approx$1L.min^{-1}) is added directly to the cone to rinse the eggs. The overflow water is removed from the cone by a permanent siphon with the intake screened to retain the eggs in the cone (Fig 13). Rinsing is continued until the water is clear, which can take up to 60 minutes.

Figure 12:   Egg counting cone

Once rinsed, the siphon is removed and the eggs are aerated vigorously to ensure uniform distribution. A minimum of six 5ml samples are then taken from the tank using an autopippette. Each sample is dispensed onto a taut piece of 500 μm mesh where the eggs can be seen and counted. The six counts are averaged, then divided by 5 to obtain the average number of eggs per millilitre. To obtain the total number of eggs in the cone, this value is then multiplied by the volume of the tank.
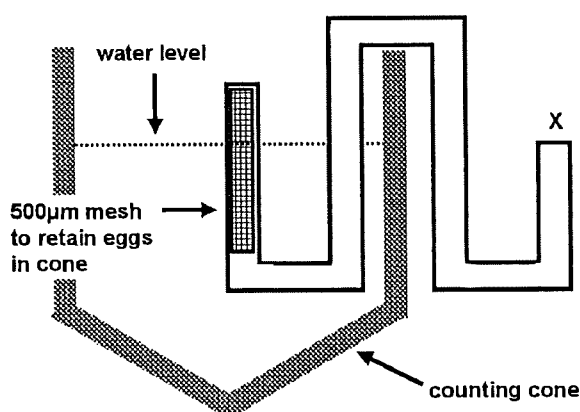
Figure 13:   Permanent siphon. If the siphon is completely filled with water (ie no air bubbles) then it will continue to remove excess water whenever the water level rises above the top of the outlet column (marked X).

5.7 Egg quality

Rearing marine fish larvae requires expenditure of time, effort and money. As larval survival and quality are highly dependent on egg quality, only the highest quality eggs are selected to maximise production efficiency. At the ADU, three methods are used to evaluate egg quality, namely buoyancy, fertilisation rate and early larval survival.

The first method relies on the premise that good-quality eggs, having a high lipid content, are positively buoyant, whilst those that sink are infertile, over-ripe or immature (Foscarini, 1988). In order to calculate viability, the total egg number is firstly determined, as described above. The air-stone is removed from the counting cone and the eggs left standing for approximately 20 minutes. The good-quality eggs float whilst the poor-quality eggs sink to the bottom of the tank. The negatively buoyant eggs are then removed by opening the valve at the bottom and draining at least 10L of water. The air-stone is then added and the counting procedure is repeated to determine the number of viable eggs. Percentage viability is calculated using the following equation:

$$V = \frac{N_V}{N_T} \times 100\% \qquad \text{Where:} \qquad \begin{array}{l} V = \text{viability (\%)}; \\ N_V = \text{number of viable eggs}; \\ N_T = \text{total number of eggs} \end{array}$$

If the viability of a batch of eggs is less than 80%, the quality of the eggs is likely to be inadequate for cost effective larval rearing. To confirm the quality of the eggs, an estimate of the fertilisation success can be made using a dissecting microscope. Fertilisation can be detected within two hour of its occurrence, when cell division is at the 2 or 4 cell stage. For an accurate measurement it is best to wait a further 6-12 hours before checking. By this stage the developing embryos will be clearly visible. Infertile eggs, on the other hand, will show no development, or development will have halted at an earlier stage of cell division. A good batch of eggs will have a very high fertilisation rate, usually greater than 90%.

Larval survival during the first two days post-hatch is perhaps the most accurate and reliable indicator of egg quality. Good-quality eggs provide the developing embryo with enough nutrients and energy to fuel early development until exogenous feeding commences. If newly hatched larvae from poor-quality eggs are stocked directly into larval rearing tanks, they may suffer high mortalities before exogenous feeding commences, due to a lack of endogenous reserves. High, early mortalities may be difficult to determine, especially in green-water cultures where small larvae can be difficult to see. It may be several days or weeks before heavy mortalities are evident, by which time substantial staff resources and funds have already been invested in the culture. At the ADU, dhufish larvae are maintained in the egg incubators (Fig 14) until the second or third day and an estimate of survival made before they are stocked into rearing tanks. If good-quality eggs are stocked into the incubators, survival to

Day 2 (where Day 0 is the day of hatch) should be greater than 50%, and the presumably good-quality larvae can be stocked into the rearing tanks.

Viability, fertility, hatch and survival to day 2 of dhufish eggs/larvae are highly variable (Appendix G and H). Egg viability can be as high as 95%, however it is more common for a viability of 50%. Mean viability of eggs stripped from acclimated females treated with LHRHa at $50ug.kg^{-1}$ was approximately 90% on the first ovulation, however, decreased with successive ovulations, and was 40% by the third ovulation (Appendix H). Fertility (expressed as a percentage of viable eggs) is normally low, being less than 60% on most occasions, however may be as high as 95%. Hatch (expressed as a percentage of fertilised eggs) is also low, with percentages of 50% or less being common, although hatch rates of 80% have been observed.

5.8 Incubation, larval harvesting and counting

Following counting, eggs are transferred to the incubation cone. The ADU incubation cone is a 40L cylindro-conical fibreglass cone with 350μm screened windows in the vertical walls. The cone has in-built buoyancy and floats inside a larger tank filled with seawater (Fig 14). To eliminate the need to later transfer the larvae to a new tank, the incubation cone should be floated in the larval rearing tank if possible. Very light aeration (sufficient to gently maintain eggs in suspension) is supplied directly to the counting cone through an open-ended airline attached to the base of the cone. Incoming water, filtered to 10μm (nominal), is passed though a degassing column and fed into the tank. The flow rate is set at 6 $L.minute^{-1}$. Water will diffuse through the mesh into the counting cone and so dilute waste products. Water overflows from the larval rearing tank through a 250μm screen.
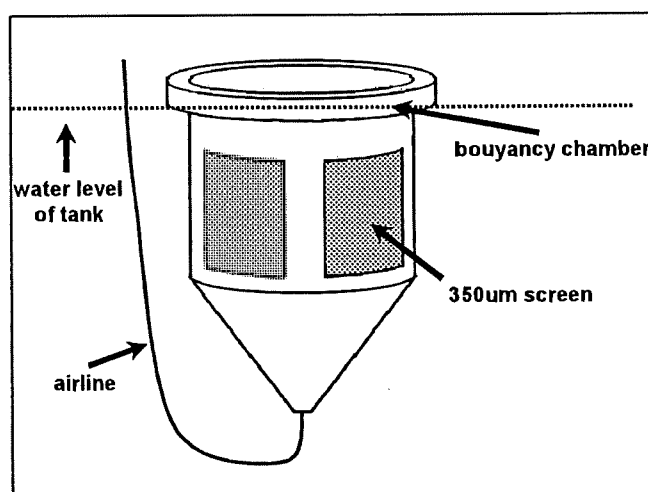
Figure 14:   Floating incubation cone

The egg transfer from the counting cone to the incubation cone is conducted by turning off air and water to enable buoyant eggs to float to the surface. The eggs are then ladled carefully off the surface using a 1L beaker and transferred directly to the incubation cone. Each incubation cone is stocked with a maximum of 2000 eggs.L^{-1}. During incubation, the tanks are kept in the dark (ie covered with black plastic or incubated in a darkened room). Hatching time is dependent on temperature; at 23°C, hatching occurs within 32h. "Dead" eggs are removed regularly (at least twice per day). This is done by turning off the air for 5 to 10 minutes. Dead eggs will settle to the bottom, while good eggs will move to the surface. The dead eggs can then be vacuumed up using a small siphon (eg a length of airline attached to a rod).

On hatch and on day 2 or 3 post-hatch, the larvae are counted. First the air is turned off to allow all dead larvae, eggs and waste to settle. These are vacuumed up using a small siphon as above. When the cone is clean, aeration is increased to aid even distribution of the larvae within the cone. Dhufish larvae are strong swimmers and so gentle stirring using a plastic paddle may also be necessary to ensure even distribution. The period of mixing is kept to a minimum to avoid damaging the fragile larvae. The effect of counting in this manner on larval survival has not been determined. At least six, 10 ml samples are counted in a petri dish to estimate the total number of larvae in the cone (by calculating average number of larvae per millilitre, and multiplying by the volume of the cone). The larvae are then stocked directly into the larval rearing tank by gently submerging the lip of the incubation cone and allowing the larvae to flow out.

It may be inconvenient at times to float the incubators within the larval tank itself. In such cases, the larvae must be transferred from the incubating tank to the larval rearing tank on day 2 or 3 post hatch. The first step is to ensure that the water quality parameters (especially water temperature), of each tank are as similar as possible to minimise shock. The air and water are then turned off to the incubation cone, to encourage the larvae to swim to the surface. The larvae are then carefully ladled off the surface in 1 L or larger beakers. The full beakers are then carried and stocked into the larval rearing tank. To minimise stress, the beaker should be floated in the larval rearing tank and the water in the beaker changed gradually over a period of 10 minutes. The larvae are then released into the tank by submerging the beaker and allowing larvae to flow out. Stress can be further minimised by conducting the transfer in dull light.

Chapter 6: Larviculture

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6.1 Overview

Rearing marine finfish larvae requires close attention to a number of important parameters including temperature, salinity, pH, tank design, lighting and the size and nutritional composition of the food that is offered. In general, larval rearing methods for marine finfish can be classified into three categories: intensive, semi-intensive and extensive.

Standard intensive methods involve rearing larvae at high densities in flowing, clean seawater. Sophisticated filtration equipment and precise temperature and lighting control are often used to maintain hygienic conditions and control over the rearing process. Effective intensive methods are comparatively expensive in terms of labour and capital. Adding marine microalgae to the larval tank is a popular adaptation to the standard intensive technique. In addition to the more obvious benefits, such as buffering water quality changes and providing a food source for the zooplanktonic prey organisms in the tank, microalgae are also reported to have benefits such as stimulating enzyme synthesis and acting as an immunological stimulant (Lavens et al., 1995).

A less-demanding method of rearing larvae is extensive culture, a method in which eggs or newly hatched larvae are stocked directly into a large tank or pond. The larvae feed on naturally occurring plankton, which proliferate with the addition of inorganic and/or organic fertilisers. Although much cheaper than intensive methods, extensive production can be unreliable due to the dependence on natural plankton blooms and poor or non-existent control over climatic conditions. Growth of larvae in extensive culture is usually excellent, however survival can be inconsistent. Predatory insects, invertebrates and other fish larvae can have a significant impact on survival.

Semi-intensive techniques have attempted to combine the positive features of both intensive and extensive methods. Rearing occurs in tanks with no constant flow and limited water additions/exchanges (called 'static' tanks), in which rotifers are encouraged to proliferate by the regular addition of microalgae. These techniques provide more control over the rearing process than extensive methods and are less costly than standard intensive methods. During 1997/98, a rearing strategy originally developed for barramundi culture (Palmer et al, 1992) was modified at the ADU for improved larviculture of black bream (Partridge et al, 1998; Jenkins et al 1999). Under this semi-intensive green-water system, larvae are reared in static (no flow) 5,000 litre tanks. Rotifers are encouraged to proliferate within the tank by the regular addition of the green microalgae Nannochloropsis oculata. Rotifers receive no artificial enrichment and feed only on the microalgae present in the culture. This method resulted in

survival rates of black bream larvae in excess of 80%, compared to an average of 20% under the previous green-water intensive culture system. For a thorough description of the various rearing methods refer to Jenkins *et al* (1999) and Partridge *et al* (1998).

A range of different larval rearing strategies have been trialed to determine the optimal rearing methods for the WA dhufish (Table 1). The rearing strategies tested include clear-water intensive, green-water intensive, green-water semi-intensive (with and without cultured calanoid copepods) and brown-water semi-intensive. These cultures have been conducted in a variety of tank sizes and styles ranging in volume from 140 litres to 5,000 litres. Survival ranged from 0 to 37%. In terms of survival, the most successful system incorporated cultured copepods, with survival being at least 12 times higher than for other methods. That trial, however was conducted on an experimental scale, and must be scaled up to be commercially viable. In terms of labour, the preferred method was the semi-intensive green-water method. Research is therefore currently under-way at the ADU to incorporate cultured copepods into a semi-intensive green-water system, thereby combining the nutritional advantages of copepods with the cost-effective semi-intensive system.

6.2 Evolution of a suitable larval rearing system for dhufish

The clear-water intensive method was first trialed in 1995/96 and 1996/97. The perceived benefits of clear-water intensive culture are hygiene, high water quality and complete control over the rearing environment. In the clear-water intensive method trialed for dhufish at the ADU in 1995/1996, larval rearing tanks received a constant flow of clean water filtered to $1\mu m$ absolute by cartridge filtration and sterilised by ultraviolet radiation. Water temperature was maintained between 22 and 24 °C. Larvae were fed rotifers (*Brachionus plicatilis*) at first feed. Later they were weaned to early developmental stages of brine shrimp *(Artemia* sp). The rotifers and artemia were enriched prior to feeding with various artificial enrichment or algae. A total of 18 trials were conducted using this method. Only one of these batches was successful in producing juveniles and survival was low with a maximum survival (to Day 50 post hatch) of 0.01% (Table 1).

Due to the poor results obtained, green-water intensive methods were trialed and microalgae was incorporated into the rearing tank. In the green-water intensive method, larval rearing tanks received a constant flow of clean filtered water as per the clear-water intensive method. In addition, approximately 500,000 cells.mL^{-1} of the green microalgae, *Nannochloropsis oculata (N.Oculata)* was maintained within the tank during the rotifer-feeding phase. Feeding was as for the clear-water intensive method. This method was attempted on a total of eight batches in 1996/97 and 1998/99. Success was again limited with only one batch producing juveniles (survival 0.01% to day 50 post hatch).

Due to the success of the semi-intensive method to rear black bream, this method was trialed on seven batches of dhufish larvae in 1998/99. Methods were similar to that described for black bream in Jenkins *et al* (1999) and Partridge *et al* (1999). Briefly, a 5000L tank was partly filled with 1200L of 5μm filtered seawater. The water was chlorinated and neutralised before the alga *N.oculata* and rotifers (40.ml^{-1}) were added. The larvae were added at a density of 30,000 to 100,000 larvae per tank. Additions of algae and seawater were made regularly in order to maintain suitable water quality parameters. When the tank was full, a water exchange of at least 500L.day^{-1} was made to control water quality parameters. Feeding was as for the clearwater intensive system with two exceptions: 1) Rotifers were not artificially enriched as rotifer populations were self-maintained in the tank and no rotifer additions were required throughout the trial; 2) The culture system promotes blooms of other zooplankton, including wild copepods which add to the available food source. The semi-intensive method generally requires less maintenance than for the intensive method and the enrichment and daily addition of rotifers is generally not required during the first two weeks of culture. Due to the more static nature of these systems, water quality is of critical importance and more time is involved in monitoring essential water quality parameters than for intensive methods. Results were variable, however, the maximum survival to Day 50 post hatch was 3%: a result manifold higher than previously obtained for dhufish in any culture system.

As dhufish are a deep water, oceanic species it was considered likely that they would have a higher requirement for highly unsaturated fatty acids (HUFA), compared to the estuarine black bream. *N.oculata*, the background algae species used in standard greenwater cultures at the ADU is low in the HUFA, docosahexaenoic acid (DHA). Therefore a variation was trialed by replacing *N. oculata* with Tahitian *Isochrysis galbana* (T.*Iso*). This culture also resulted in superior survival compared to all previously attempted intensive culture techniques (survival to Day 50 was 1%), but slightly lower than obtained in the standard green-water semi-intensive culture.

At the same time, a highly successful adaptation of the green-water intensive method was developed (1998/99), utilising cultured calanoid copepods (*Gladioferens imparipes*) during the rotifer feeding stage (Rippingale and Payne, in review; FRDC Project # 96/398; Payne *et al*, 2000). The algae, (T.*Iso*) was added in addition to *N.oculata* as enrichment for the copepods. This was trialed on an experimental scale in 140 L tanks and compared with larvae in similar tanks which were fed rotifers only. Survival in the copepod-fed tanks was high, being 37% to day 50 post hatch, compared with 5% in the tanks fed rotifers only. In addition, growth was significantly higher in the copepod-fed tanks, achieving a length of 11mm by day 23 compared with only 6mm in rotifer only tanks (Payne *et al*, 2000). This trial also demonstrated that dhufish larvae could be reared in small experimental scale tanks. This is an important development as it enables replicated studies with small numbers of larvae.

Table: 1   **Summary of larviculture trials**

Year	Culture Method	Number of batches	Food presented at first feed	Artificial rotifer enrichment ?	Background algae	Tank Size (L)	Maximum Survival to Day 50
1995/96	Clear-water intensive	8	Rotifers	Yes	N/A	300 – 2,000	0.01%
1996/97	Clear-water intensive	10	Rotifers	Yes	N/A	1,000	0%
	Green-water intensive	2	Rotifers	Yes	*N. oculata*	1,000	0.01%
1998/99	Green-water intensive	6	Rotifers	Yes	*N. oculata*	1,000	0%
	Green-water semi-intensive	7	Rotifers & naturally occurring copepods	No	*N. oculata*	5,000	3%
	Brown-water semi-intensive	1	Rotifers & naturally occurring copepods	No	*I. galbana.*	5,000	1%
	Green-water semi-intensive	3	Rotifers	No	*N. oculata & I. galbana*	140	5%
	Green-water semi-intensive	3	Rotifers & cultured copepods	No	*N. oculata & I. galbana*	140	37%
1999/2000	Green-water semi-intensive		Rotifers & cultured copepods	No	*N. oculata & I. galbana*	140-5000	10%
	Green-water aquamat semi-intensive	1	Rotifers & naturally occurring copepods	No	*N. oculata & I. galbana*	5000	0%

Despite the advantages of cultured copepods to the larvae, the system was conducted in small scale (140L) tanks and is too labour intensive to be commercially viable. Therefore, a system that combines the benefits of cultured copepods with the labour saving commercial scale semi-intensive green water system is now being developed. Insufficient production of cultured copepod nauplii is currently a bottleneck to developing such a system. Therefore a system that promotes a self-sustaining population of cultured copepods within the larviculture tank is being investigated. This would eliminate the need to add copepod nauplii to the tank daily, and so would decrease the total number of intensively cultured copepods required. Preliminary trials, however, have indicated that the high densities of *N. oculata* typical to the standard static green-water system were too high for calanoid copepods, resulting in low copepod survival and nauplii production. Further development of this system and optimisation of techniques for calanoid copepod culture is being funded by a grant from the Aquaculture Development Council (1999/2000).

Due to the uncertainties of achieving sufficient copepod production for commercial dhufish larviculture, research is continuing to maximise survival and growth of dhufish larvae fed solely on rotifers. Dhufish larvae are capable of efficient ingestion and digestion of rotifers. The nutritional content of rotifers can be manipulated by adjustments to enrichment protocols. Therefore a better understanding of the nutritional requirements of dhufish larvae, and subsequent improvements to rotifer enrichment protocols, should result in improved survival, growth and larval vigour.

6.3 General larviculture principles

The following are some general comments on larval rearing techniques as they apply to dhufish culture. For a more complete discussion of these and other aspects of marine larval rearing refer to Jenkins *et al* (1999) and Partridge *et al* (1999).
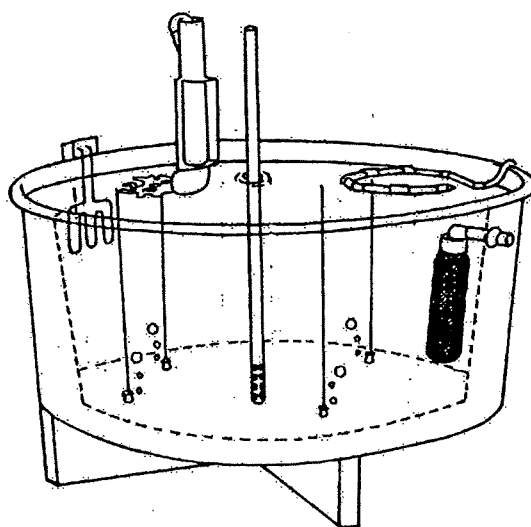
Figure 15:   5000L larval rearing tank

A range of tanks have been used for rearing dhufish larvae ranging in size from 140 to 5000L. All semi-intensive methods have been conducted in 5000L tanks (Fig 15). The bottom of all the tanks are white and the sides either dark blue or black. Tanks with dark-coloured walls are reported to be superior to those with white walls, as the dark colour enhances the visibility of the prey to the larvae. The white bottom enables the culturist to see the larvae and the bottom of the tank. A cylindro-conical tank is preferred due to the superior tank dynamics.

Although it is possible to follow a 'recipe' for culturing marine finfish larvae, successful results will only be obtained through close observation of the culture and the larvae themselves. Monitoring the progress of the developing larvae, by microscopic observation, is a very important aspect of larval rearing. Important information such as feeding response, growth rates and swim bladder inflation can all be gathered from these observations. At the ADU, observations are made by selecting and examining approximately ten larvae from the tank every day during such times as first rotifer feeding, swim bladder inflation, and rotifer/artemia weaning, and on alternate days outside of these periods.

Food consumption is quantified by assigning a 'score' of 0, 50 or 100% to the fullness of each larva's gut. These observations are particularly important at first feeding and during the transition from one food type to another, because they provide an indication of how well larvae are adapting to the new food type.
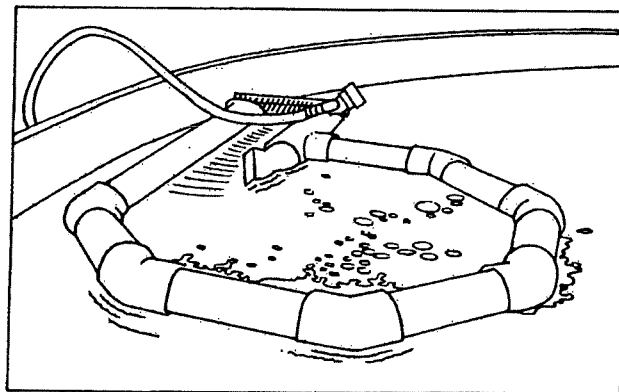
Figure 16:   Surface skimmer

Growth is assessed by measuring the standard length of the larvae using an ocular micrometer fitted to the eyepiece of a dissecting microscope. Larval growth is highly dependent on temperature and food availability. Newly hatched dhufish larvae are 1.75-2.10 mm in body length (Pironet and Neira 1998; Appendix M)). As a guide, larvae should reach 5 mm by Day 15 and 10 mm by Day 30.

A critical stage of larval development of many cultured species is swim bladder inflation. Larvae initiate swim bladder inflation at night by 'gulping' air from the surface and passing it through their pneumatic duct into their swim bladders. This process can only occur within a narrow window of time. An oily film on the water surface increases the surface tension, which

can prevent the small larvae from breaking through and gulping air. Surface skimmers are therefore added to the larval rearing tank from Day 2 to remove surface film (Fig 16). The skimmers operate by passing a stream of air across the surface of the water into an enclosed area, in which proteins and oils accumulate and are periodically removed. Larvae with inflated swim bladders have an air-filled cavity above the stomach and are easily identified under the microscope.

Swimbladder inflation in dhufish begins on about Day 3 or 4 for dhufish. The proportion of fish with evidence of swimbladder inflation should be determined daily during this period, as this gives an indication of the health of the larvae and whether skimmers are operating effectively. Dhufish larvae have not demonstrated difficulty in inflating swim bladders in tanks fitted with surface skimmers. In all trials conducted so far, 100% of larvae have inflated swim bladders by day 8 post-hatch.

6.4 Summary of developmental stages

Dhufish larvae hatch within 32h of fertilisation at 23°C (Fig 17). Yolk sac larvae are 1.75 to 2.10 mm long (Fig 18). First feed occurs on day 3 or 4, at a length of 2-3mm. Swimbladder inflation occurs simultaneously, with 100% inflation occurring between Day 3 and 8, and at a length of 2 to 4mm. Artemia feeding commences at a larval length of approximately 5mm. Metamorphosis is complete by Day 40 to 45, and at a larval length of 8mm (Fig 19; Pironet and Neira, 1998; Appendix M).
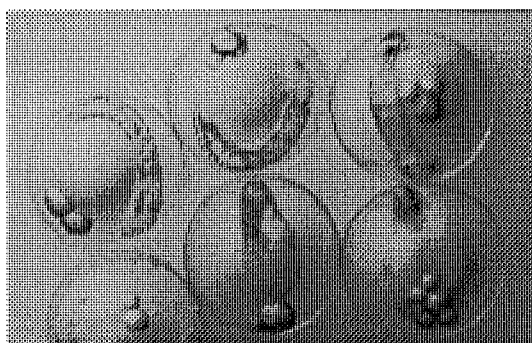
Figure 17:   Dhufish eggs undergoing   Figure 18:   Newly hatched dhufish
               organogenesis   larvae
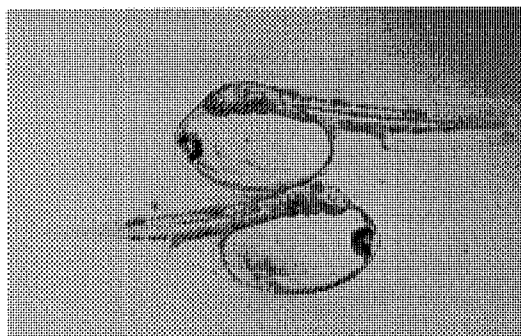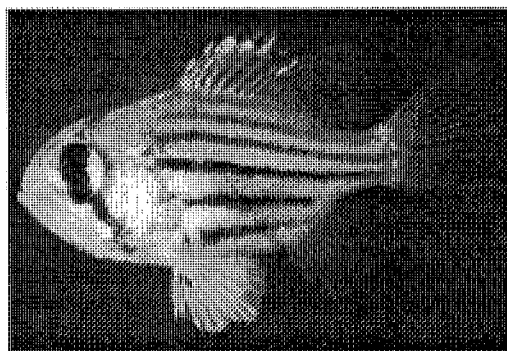
Figure 19:   Fully metamorphosed juvenile dhufish (Photo courtesy of Barry Hutchins)

6.5 Weaning to a pellet

Weaning involves a gradual change in the primary food source from live artemia to an inert pellet. At the beginning of weaning, artemia levels are maintained, and small amounts of the pellet are added regularly. Gradually the amount of pellet is increased, while the amount of artemia is decreased. The reduction in available artemia encourages the larvae to accept the pellet. For a weaning diet to be effective it must be readily ingested, highly digestible and contain the correct balance of essential and non-essential nutrients. The standard weaning diet used at the ADU for black bream and pink snapper is an imported formulation, known as Nippai ML. This is a highly effective diet for weaning black bream and snapper. Initial attempts to wean dhufish using this standard technique were unsuccessful, as the dhufish did not accept the diet.

To identify a successful weaning strategy for dhufish, a series of trials were conducted comparing various methods of presenting the artificial pellet (Nippai ML). In one trial three different weaning diets were used: 1) Nippai ML; 2) Nippai ML coated with fish emulsion and 3) a gelatin-based pellet containing Nippai ML and fish flesh. The first two diets were used throughout the entire weaning period. In the third diet, the proportion of flesh was initially high, and the Nippai ML was low. Gradually the proportion of fish flesh was reduced to 0%, while the proportion of Nippai ML was increased to 100% (Appendix Q). The acceptance of Nippai ML and coated Nippai ML (first two treatments) was poor, and groups of fish weaned straight onto this pellet suffered high mortality and slow growth. Weaning using the gelatine-based pellet was more effective than both Nippai ML and coated Nippai ML, with a lower mortality (10% cf 40%). The superior performance of the gelatine-based pellet, compared with the commercial diet appears to be due to increased attractiveness to the fish. Preparation of the gelatine pellet, however, is labour intensive.

To date, only a slow wean (over 30 days) has been attempted with dhufish and only fully metamorphosed juveniles have been tried. Weaning of marine fish is normally conducted at a much younger age, and over a shorter period of time than has been successful for dhufish (see Jenkins et al, 1999). Trials will continue in order to develop a less labour intensive method of weaning, to reduce the period of the wean and to successfully wean at a younger age.

6.6 Harvest and survival estimates

At the ADU, marine fish larvae other than dhufish are routinely harvested by feeding water, via gravity, through the bottom drain of the larval tank into a reservoir fitted with a suitably sized net. Various methods are in use or are being developed at the ADU for estimating larval survival with marine fish larvae. These methods include estimating by volume when larvae are concentrated in a small volume of water both with and without anaesthetic (similar to estimating for hatchlings) and the use of a fish counter (which counts each larva as it passes through a beam of infra-red light). Refer to Jenkins et al (1999) for more details.

Due to the comparatively small number of dhufish cultured to date, these methods for harvest and estimating survival have not been fully explored for their suitability for use with dhufish larvae. The only harvest method used routinely on dhufish has been to drain the tank to a depth of 20 cm and scoop out fish, a few at a time, using a net or small beaker. Fish are then counted as they are transferred to a new tank. This is a highly accurate but time consuming method, particularly for large numbers of fish. Furthermore, it has only been performed on fully metamorphosed fish and would not be suitable for younger larvae. Other methods for harvesting and estimating survival will be trialed on dhufish in the future.

Chapter 7: Nursery and grow-out

Since dhufish culture is in its infancy, very little information is available in regard to optimal diets, conditions or systems suitable for grow-out. To date, less than 2000 dhufish have been grown in captivity beyond the nursery stage. Improvements in larval rearing technology, however, have resulted in increased numbers of juveniles available for investigations into optimal conditions for grow-out.

7.1 Husbandry

Juvenile dhufish have been held in 300-25,000L tanks (depending on the number of fish), supplied with aeration and with filtered seawater. Dhufish have not been trialed in seacages. The effect of stocking density on the growth rate of juvenile dhufish has not been investigated. Dhufish juveniles are currently held at stocking densities of up to 4kg.tonne^{-1}. Normal temperature range is 17-22 °C. As dhufish are prone to jumping when startled, all tanks must be covered with a well secured net. For this reason tanks are preferably located in low-traffic areas. Cylindro-conical tanks are preferred for ease of tank maintenance. Tanks are vacuumed and the drain valve purged daily to remove faeces and waste food.

7.2 Grading

As with all marine finfish, growth rate can vary greatly between individuals. This can cause problems in the nursery as larger fish can show aggression to smaller fish and out-compete them for food. Although dhufish are not aggressive or cannibalistic, it may be necessary to grade them during their commercial culture to reduce competition and ensure equal access to food.
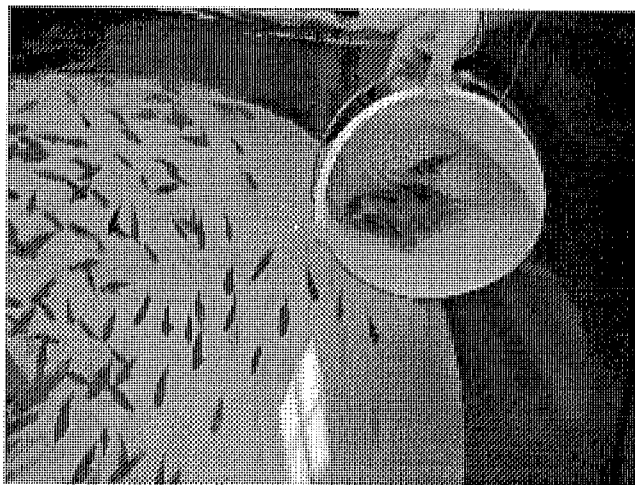
Figure 20: Juvenile dhufish being stocked into a new tank following grading

A passive grader may be used for grading dhufish. This is a large box with equally spaced metal rods in the base. An appropriately spaced grid should be selected to ensure the desired grade. Unlike snapper and black bream, dhufish do not swim down through the grid, therefore, the grader must be completely lifted out of the water briefly to encourage the smaller fish to pass through. The larger fish are then retained in the grader and are transferred to another tank or tub (Fig 20). Refer to Jenkins *et al* (1999) for more details.

7.3 Feeding and nutrition

Little is known about nutritional requirements for juvenile dhufish. Many health problems are evident in the juveniles that appear to be a consequence of inadequate nutrition. The most distinct of these is "fatty liver" syndrome. Although many cultured species exhibit fatty livers, the severity of the problem in dhufish is extreme. The problem first became evident in fish fed on a commercial pellet (protein:energy ratio of 40:15). Severe fatty livers, coupled with low, but constant mortality led to all fish being fed a flesh diet (supplemented with vitamins and minerals). The change in diet temporarily abated the mortalities, however, the livers did not improve and the fish remained plagued by poor health. At the time, the fish were being fed a rotational diet of thawed, freshly frozen pilchards, prawns, squid and whitebait. In an effort to reduce the fat in the diet, pilchards were replaced by garfish, mullet and whiting. Recent liver histology from juvenile dhufish has indicated that the fatty livers are substantially improved. It is likely that the change in diet has contributed to this improvement.

The current diet for dhufish juveniles at the ADU is now a rotational diet of garfish, mullet, whiting, whitebait and prawns, all chopped to an appropriate size. Fish are fed to satiety once to three times daily, depending on the size of the fish. Satiety for dhufish held at the ADU is 3% of body weight (wet weight). Dhufish juveniles also readily accept a moist pellet. The pellet is adapted from Glencross *et al* (1999) and contains 30% flesh in a gluten base (Appendix D). Fish are fed the moist pellet twice per week. This enables the diet to be supplemented with additional vitamins and/or therapeutics.

When the juvenile dhufish were fed on the commercial pellet, acceptance of the pellet was good, although feeding was less vigorous than for flesh-fed fish and growth appeared slower. Dhufish juveniles fed on pellets are slow feeders and are particularly well suited to an automatic feeder. Fish were fed at about 1-2% of body weight (dry pellet to wet body weight).

Investigations to define specific nutritional requirements for dhufish for the purpose of developing a suitable pelletised diet have commenced. Amino acid composition of the flesh of dhufish was found to be similar to that observed for other species (Glencross *et al*, in review; Appendix O). Native fatty acid composition, however, was significantly higher in arachidonic and decosahexaenoic acids, and lower in eicosapentaenoic acid than typically observed in standard commercial fish oils. This data suggests that standard protein

resources, provided digestible limits are observed, will suffice for this species. However, use of fish oil as the only lipid resource may not be sufficient to satisfy essential fatty acid requirements. The next step in developing a pelletised grow-out diet suitable for this species is a detailed examination of the dietary and energy requirements for this species.

7.4 Growth rates

Limited long-term data on growth of hatchery-reared juveniles has been collected. This is due to the small number of dhufish produced in the early years of the project (Cohort 1997-1). Due to their high value as future broodstock, these fish were not subjected to frequent weight-ins. Instead, these fish were weighed opportunistically whenever the fish were being handled for another reason. Recent batches of fish have been weighed more regularly (Cohorts 1999-1, 1999-2, 2000-1), however this data extends only to 400 days, due to the age of the fish. Wet weight of these four different cohorts of hatchery-reared dhufish are shown in Figure 21.

Growth in three of the four cohorts (1999-1, 1999-2, 2000-1) followed a similar curve. These fish reached (or are expected to reach) approximately 100g in 400 days. The weight of the fish in cohort 1997-1 was significantly higher than this at approximately 400 days with fish having attained 280g; almost three times that of the other three cohort. The rearing conditions between the faster growing 1997-1 and the other three cohorts varied considerably.

- Water temperature: Average temperature for the 1997-1 fish over the first 400 days was $22.4°C \pm 0.5$, while in the other fish it was $20.5 \ 4°C \pm 0.5$; a difference of $2°C$. The effect of temperature on growth has not been investigated for dhufish, however, these data suggest that the effect may be considerable.
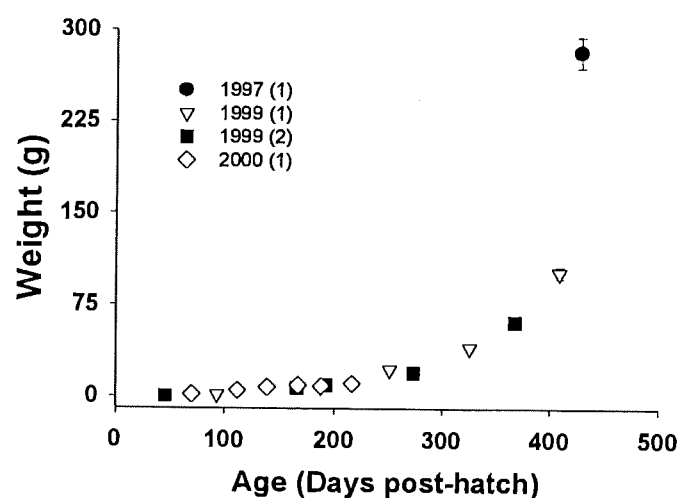
Figure 21: Wet weight of dhufish juveniles from four different cohorts of eggs. Values are means ± SE.

- *Stocking density*: 1997-1 fish were held in a 25,000L tank and at day 400 stocking density was only 1.33 g.L^{-1} (based on 95 fish at 280g each). In contrast, the other three cohorts were held in much smaller 2,500L tanks and at 400 days, stocking density was approximately 8g.L^{-1} (based on 200 fish at 100g each); a 6-fold difference in stocking density. It is possible, although unlikely that this higher stocking density may have impaired growth. Optimum stocking density has not been investigated however even 8g.L^{-1} is not high for marine finfish grow-out, and so is thought to be less significant than water temperature.

In one year (ie 365 days), dhufish in the wild reach approximately 100mm (Hesp and Potter, 2000), which corresponds to approximately 27g wet weight. Therefore growth of dhufish in even the slowest growing hatchery-reared cohorts was more than 3 times faster than in the wild. The increased growth rate exhibited by the hatchery-reared fish is likely to be due to the increased quantity of food available, reduced foraging effort and presumably increased temperature, although the preferred temperature of juvenile dhufish in the wild is unknown.

Chapter 8:   Health and disease management

Co-author Dr Fran Stephens, School of Veterinary Studies, Murdoch University.

In the wild, dhufish live in waters deeper than 10 metres and with a temperature range of 19 to 22°C (Pearce, 1991). In the culture environment dhufish are held in conditions that are vastly different to their natural habitat: tanks are shallower (from 1 to 3m in depth), temperature variations are broader (16-26°C), stocking densities are higher and the diet is different. These, in addition to daily activity and routine hatchery procedures, combine to impose considerable stress on captive wild dhufish, which may manifest itself in diseases and other health problems.

The three most common health issues encountered with captive dhufish are 1) exophthalmia ("pop-eye"), 2) heavy gill fluke infestations and 3) various bacterial, fungal and protozoan infections. Dhufish appear to be sensitive to many chemical therapeutics normally used on other cultured fish. This factor makes treatment of some diseases more difficult. Issues relating to dhufish health are currently under investigation by Dr Fran Stephens of the School of Veterinary Studies, Murdoch University (FRDC Project #98/328).

8.1   Disease recognition

Early recognition of potential disease problems is important in diagnosing the cause of the disease and implementing appropriate treatments. Careful observation is the best way to recognise disease problems. Signs that fish are unhealthy or stressed include:

- Dark colouration

- Weight loss (cranial bones more obvious than usual)

- Abnormal behaviour eg reduced appetite, fish remaining alone in a dark part of the tank.

- Red or swollen areas on the body of the fish

- Tattered fins and/or tail

- "Flashing" or rubbing against tank surfaces

- Increased ventilation

- Stationing of the fish at air sources or water inlets

8.2 Disease diagnosis

It is important to diagnose the cause of a disease problem before starting treatment. All handling and treatments will further stress the fish and may result in increased problems if the treatments are inappropriate.

The first steps to diagnosing health problems with dhufish are to anaesthetise the affected fish and take a gill biopsy and skin scraping. A gill biopsy involves sampling a small portion of the gill filaments. Sterile surgical scissors should be used and the gills placed in a vial containing seawater. Some bleeding may occur from the gills. The gill filaments and skin scrapings are then observed on a glass slide under a microscope. Motile protozoan parasites and gill fluke will be visible under low power if the fish is heavily infested. For a comprehensive description of these techniques and of the organisms likely to be found, see the Fisheries WA publication "Fish Health for Fish Farmers in Western Australia" (Thorne, 1995).

For further diagnosis, live or freshly dead fish can be submitted for a full post mortem and further tests to the Fish Pathologist at the Fisheries WA Fish Health Unit located at Agriculture WA in South Perth or the equivalent agency in other states. If it is not possible to submit a whole fish the following procedures may help to obtain a diagnosis.

- Skin lesions can be swabbed with a sterile swab, held on ice and submitted for bacterial and fungal culture.

- Pieces of gill and internal organs up to 1.5cm in diameter can be removed from freshly dead fish, placed in 10% buffered formalin and submitted to the Fish Pathologist.

8.3 Identified diseases and their treatments

8.3.1 Infectious diseases

Gill fluke

Two gill fluke species have been identified in dhufish. The most prevalent one is *Haliotrema* sp. The other is an unidentified member of the subfamily *Axinae*. This later parasite has not been detected in the previous 3 years of the project. *Haliotrema* sp is the most problematic pathogen of captive dhufish. Infested fish become dark in colour, station themselves above airstones and their rate of opercula movement increases. Fish infested with gill fluke frequently "flash" (turn sideways and rub flank on the floor of tank or on piping). Populations of gill fluke in captive fish increase with increasing water temperature, hence most problems occur during late spring and summer if the infestation is not controlled.

Haliotrema are very resistant to many treatments, and so far a 100% effective treatment has not been identified. The margin of safety between an effective dose and a safe dose is very narrow for many common treatments. To date, a freshwater bath has been the only effective treatment. Recent trials, however, conducted under FRDC project 98/328, have identified a range of potential treatments including trichlorphon, praziquantel, toltrazuril and formalin. These treatments will be further investigated and reported at the completion of that project.

<u>Freshwater bath</u>

This treatment is routinely administered to captive dhufish, newly arrived to the hatchery, during the quarantine period and 2 times per year as a prophylactic control measure (and at other times if required). This treatment is effective in removing isopods and copepods from the skin and nares and removes the majority of gill fluke. The treatment is presumed to be not effective against eggs and cysts so, it is repeated after 14 days to prevent reinfection by newly hatched parasites. The freshwater bath is used successfully on both broodstock and juveniles, although extra care is taken with the small fish due to their increased susceptibility to osmotic stress.

The treatment is administered in a small volume of water (250 to 1500L), to maximise the rate of water exchange and therefore the rate of salinity decrease. At the ADU floating crowds, 250L-1000L tubs and 4000L tanks with lowered water level have been used. Fish undergoing quarantine are normally treated in the 4000L quarantine tank. Fish in large broodstock tanks are lightly anaesthetised with AQUI-S (0.2 ppm) and transferred to a 250 to 1000L tank for treatment. Floating crowds can be used for treatment, however, are no longer the preferred vessel. While the treatment is underway the broodstock tank is cleaned and then refilled. This is easier to do if the fish are first removed from the tank, rather than left in a crowd floating in the same tank.

Once the fish are in an appropriate vessel, the salinity is dropped rapidly down to 1 ppt (within half an hour) for 1.5h and then increased back to 35 ppt. It is critical that the salinity be measured accurately, as there is a small margin between the lethal salinity for the parasites and the fish. Oxygen is supplied throughout the treatment. Town water filtered with a charcoal filter (to remove chlorine) is suitable for the freshwater bath. In the summer the town water can get very warm (up to 30°C). Therefore prophylactic treatments are usually administered in spring and autumn, thereby avoiding summer. If a treatment is essential, the water must be cooled prior to use. At the ADU a tub is filled with water (combination of fresh and saltwater) and ice to give a salinity of 10ppt at a similar temperature to the tank. The lightly anaesthetised fish are then added to this freshwater bath. Once the fish are recovered, more filtered freshwater is added to the tub to reduce the salinity to the required 1 ppt.

During the treatment most fish will lose buoyancy (due to reduced density of freshwater compared with salt water) and become dark. Fish are monitored closely and treatment

terminated if the fish become stressed (evidenced by rapid swimming and gasping at the surface).

At the conclusion of the treatment, the fish are anaesthetised lightly again (ensuring that salinity is back up to full strength seawater) and transferred to the broodstock tank which is now clean and refilled.

Caligus

This undescribed species of *Caligus* is found commonly in wild dhufish and regularly in captive dhufish. Although outbreaks of *Caligus* are uncommon, they cause considerable irritation to the fish. Outbreaks are associated with increasing water temperature. *Caligus* is a parasitic copepod which clings to the skin on the head and the cornea of the eye (Pironet and Jones, 2000). Affected fish cease feeding and develop deep ulceration, usually on the forehead. It can be controlled effectively by freshwater bath (as for gill fluke).

Bacteria

Bacterial infections are common in captive dhufish. A number of species have been identified including *Vibrio* spp. (*V. parahaemolyticus, V. alginolyticus,, V. fluvialis* and other unidentified species; Pironet and Jones, 2000), *Photobacterium* spp (*P. damsela damsela*; Pironet and Jones, 2000) and *Flexibactor marinarum.* Few antibiotic treatments have been trialed on dhufish, however oxytetracycine has been found to be a safe and effective antibiotic. It is normally administered as a water bath at 100 ppm for 2hrs for ten successive days.

Fungi

Fungi have occasionally been observed in captive dhufish. *Exophiala* sp ("black yeast") has been the most problematic and affected 1 year old hatchery reared fish. It was successfully controlled by repeat treatment of formalin/malachite green at 75ppm.

Cryptocaryon irritans (white spot)

Only one outbreak of this protozoan pathogen has been identified over the duration of the project. It occurred in fish recently caught from the wild. After initially feeding well, the fish ceased feeding 8 days after capture and later developed vertical stripe-like haemorrhagic lesions across the flanks of the affected fish (Pironet and Jones, 2000). One affected fish died, however, after treatment with formalin/malachite green (2 treatments 5 days apart) the five remaining affected fish recovered slowly.

Aega cyclops

Although common in the nostrils of wild-caught fish, these isopods have not been observed in captive fish that have been treated during quarantine. They are effectively controlled by a freshwater bath (Pironet and Jones, 2000)

Philometra sp

The nematode parasite Philometra sp. is common in the ovaries and testes of dhufish in the wild. There are three main stages of infection; larvae, adult worms and a granuloma. Although most captive dhufish examined have granulomas (evidence of previous infection) active Philometra has not been detected in fish that have been in captivity for more than 1 spawning season. This is most likely due to the absence of a secondary host in the culture environment (A. Hesp, Murdoch University, 1999 pers com). The persistence of the necrotic mass in the gonads for several years following the last active infection is, however, a problem for the culture program. In most cases the necrotic mass concentrates in the posterior end of the oviduct. In several cases it has caused difficulties in conducting ovarian biopsies and in stripping eggs. The effect on spawning fish is unknown, however, it is possible that the necrotic mass may impede the release of eggs from the oviduct. No treatment to break down the granuloma has been developed.

8.3.2 Non-infectious diseases

Exopthalmia

Exophthalmia is a common condition amongst captive dhufish and in a variety of other cultured fish species worldwide. The problem is easily identified by abnormal swelling of the eyeball, however, the cause of the condition is poorly understood at the present time. It was previously considered that exophthalmia was primarily induced by impact on the eyeball (eg during handling or if the fish strikes the side of the tank when startled). It is now considered more likely that environmental stress (eg high temperatures) and/or rapid and prolonged swimming initiates a series of physiological events resulting in gas secretion into the eye. To reduce the occurrence of exophthalmia it is recommended that peaks of high temperature, sudden noises, rapid movement and changes in light intensity be avoided as these may increase the likelihood of the condition.

There is currently no treatment for exophthalmia, except for the complete removal of the eyeball. This is a relatively simple procedure and can be done under anaesthesia. Although fish with a removed eye does resume feeding shortly after the excision, the fish do not thrive and so eye removal is seldom performed. Many fish do "recover" from exophthalmia, however, they eyes may remain damaged permanently. This represents the major impact of exophthalmia on dhufish culture. In the market, fish quality is often determined by the clarity of the eye, and a disfigured (or missing) eye would certainly reduce the value of whole fish,

limiting sale of affected fish to fillets or headed and gutted. A thorough understanding of the disease and means for prevention is therefore essential. This is the primary focus of research being conducted Dr Fran Stephens of the School of Veterinary Studies, Murdoch University (FRDC Project #98/328).

"Fatty liver" syndrome

"Fatty liver" syndrome has been identified in hatchery-reared dhufish from pre-wean onward. Although this is common in many aquaculture species, the severity of the condition in dhufish is considered extreme. The syndrome appears to be associated with atherosclerosis and/or necrotic granulomas in the liver of older fish (from 6 months of age). The condition is suspected to be of presently undefined nutritional origin (perhaps overfeeding, nutritional imbalance or deficiency of some specific dietary component/s). The resultant metabolic dysfunction appears to result in reduced growth rate, and feed conversion efficiency. In addition, most groups of fish with this problem have suffered repeated outbreaks of various diseases including gill-fluke, *Exophiala* and bacterial branchitis and septicaemia. These are suspected to be a secondary result of the underlying nutritional disease.

Chapter 9:   References

Barnetson, I.   (unpub).   Market Potential for WA Dhufish (Glaucosoma hebraicum). Independent report prepared for the Aquaculture Development Unit, South Metro TAFE.20pp

Cleary . J.J. (1997). The effect of stress o reproduction in snapper, Pagrus auratus. Thesis. Department of Aquaculture, University of Tasmania, Australia.

Cleary, J., Jenkins, G., and Pankhurst, N., (1999). Slow Release LHRHa Pellet Stimulates Spermiation and Ovulation in Westralian Dhufish (Glaucosoma hebraicum). Proceedings of World Aquaculture Society Conference, 26 April - 2 May 1999, Sydney, Australia.

Glencross.B,D., Van Barnveld, R.J., Carter, C.G. and Clarke, S.M. (1999). On the path to a manufactured feed for farmed Southern Bluefin Tuna. World Aquaculture Society Journal 30: 42-46

Glencross, B.D., Curnow, J., Aris, A. and Cleary, J. (2000). Evaluation of feed ingredient utilisation and native tissue composition as a precursor to diet development for the West Australian Dhufish (Glaucosoma hebraicum). Aquaculture in review

Hesp, S.A. and Potter, I.C. 1999. Determination of the biological parameters required for managing the fishery of West Australian dhufish.   Fisheries research and Development Corporation Project 96/103 Final Report 31 pp

Hobby, A. and Pankhurst, N.W. (1997). Post-ovulatory egg viability in the snapper Pagrus auratus (Sparidae). Marine and Freshwater Research 48:385-9

Hutchins, B. and Swainston, S. (1986). Sea Fishes of Southern Australia. Complete Field Guide for Anglers and Divers. Swainston Publishing Perth p 52-53

Jenkins, G.I., Frankish, K.R. and Partridge, G.J. 1999. Manual for the Hatchery Production of Black Bream (Acanthopagrus butcheri). Information Series 1999/1 Aquaculture Development Unit, South Metropolitan TAFE. 125pp

Lavens, P., Sorgeloos, P., Dhert, P. and Devresse, B. (1995). Larval Foods. In: Bromage, N. and Roberts, R.J. (eds), Broodstock Management and Egg and Larval Quality. Blackwell Science, Oxford. pp 373-397.

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

McKay, R. J., 1997. Pearl pearches of the world. (Family Glaucosomatidae). An annotated and illustrated catalogue of the pearl perches known to date. FAO species catalogue. FAO Fisheries Synopsis. 125(17). Food and Agriculture Organization of the United Nations, Rome, 26 pp.

Palmer, P.J., Burke, J.B., Willet, D.J. and Simpson, R.R. (1992). Development of a low-maintenance technique for rearing barramundi (Bloch) larvae. Queensland Department of Primary Industries, Information Series, No. Q192036

Pankhurst, N.W. 1998. Reproduction. Pages 1-26 *in*. K. Black and A.D. Pickering editors. Biology of Farmed Fish. Sheffield Academic Press, Sheffield.

Partridge, G.J., Ginbey, B., Kennerly, D.V. and Frankish, K. 1998. A comparison of semi-intensive larviculture techniques. Information Series 1998/1 Aquaculture Development Unit, South Metropolitan TAFE. 28pp

Payne, M., Rippingale, R.J. and Cleary, J.J (2000). Cultured copepods as food for West Australian dhufish *(Glaucosoma hebraicum)* and pink snapper *(Pagrus auratus)* larvae. Aquaculture in press Pearce, A.F. (1991). Eastern boundary currents of the southern hemisphere. Journal of the Royal Society of Western Australia. 74: 35-45

Pironet, F. N. and Jones, J. B., (in preparation). Treatment of parasites and diseases of captive Western Australian dhufish (*Glaucosoma hebraicum* Richardson). Fremantle Maritime Centre, Fremantle, Western Australia, 25 pp.

Pironet, F.N and Neira, F.J. 1998. Hormone induced spawning and development of artificially reared larvae of the West Australian dhufish, *Glaucosoma hebraicum* (Glaucosomatidae). Marine and Freshwater Research 49: 133-42

Rippingale, R. and Payne, M. (1999). Intensive cultivation of a calanoid copepod for **live** food in fish culture. Project 99/398 Final Report

Robinson, R. G., 1987. The Morphology and Histology of the Ailmentary Tract: the Dietary Preference of the Western Australian Jewfish; *Glaucosoma hebraicum.* School of Biology, Curtin University of Technology. Third Year Student Report, 46 pp.

Sudemeyer, J.E., Hancock, D.A., Lenanton, R.C.J. (1990). Biological synopsis of Westralian jewfish *(Glaucosoma hebraicum)* (Richardson, 1845)(Pisces: Glaucosomatidae). *Fisheries Department of Western Australia. Report No. 96/97.* Fisheries Research Report. Fisheries Western Australia.

Thorn, T.J. 1995. Fish Health for Fish Farmers in Western Australia. Fisheries Department of Western Australia.

Chapter 10: Appendices

Appendix A   Decompression sickness in Western Australian dhufish (Glaucosoma hebraicum)

Author: Damien Ashby

Abstract

The Westralian Dhufish (Glaucosoma hebraicum) is a highly sought after recreational and commercial fishing species in Western Australia. Current management of the Westralian Dhufish involves a bag limit and the releasing of undersized fish. However, the returning of undersized fish will be of no service to the conservation of the fishery if the fish die from the effects of the depressurisation during capture. This report describes an experiment to test the hypotheses that upon capture, Westralian Dhufish sustain damage caused by decompression sickness and that the damage sustained increases with increasing depth.

30 fish were caught from two depth categories. 15 fish were caught in water less than 20 metres (shallow category) and 15 in water deeper than 20 metres (deep category). Gross pathology examination was performed immediately after capture and samples of the heart, liver, kidney and spleen were removed for histopathology examination back in the laboratory. Acute damage was assessed based on the degree of bubble formation, clotting and haemorrhaging (symptoms of decompression sickness) in the various tissues.

Bubbles, clotting and haemorrhaging were found in all fish from both depth categories. Bubbles were often occluding blood vessels and several empty and broken blood vessels were noted. Exophthalmia and rupture of the swim bladder occurred in some of the deep-water fish. The mean damage scores for the deep-water fish were all higher than the scores for the shallow water fish. In conclusion, both hypotheses were supported in that all fish sustain damage from decompression sickness upon capture and the damage for deep-water fish is greater than the damage for shallow water fish.

Further research is required to determine the long-term survival of Westralian Dhufish after being caught and released.

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Thankyou to Franz Van der Poll for taking me fishing and for assisting me with the examination of the fish. I also thank Franz and his wife Mary for their hospitality while I was up at Ledge Point.

I would also like to thank Greg Jenkins and the Fremantle Maritime Centre for setting up this project and helping me organise the fishing.

Thankyou to the Recreational Fishing Advisory Committee for funding this project. I hope you are pleased with the results.

Thankyou to John Beasley and the Animal Science group for assisting me with transport, cameras and film.

Finally, I must thank my family and friends, especially Jessica Patterson, for supporting me and putting up with my stress.

1.   Introduction

The Westralian Dhufish (*Glaucosoma hebraicum*) is a native to Western Australia and is one of the most sought after commercial and recreational fishing species (Figure A3).   Their distribution ranges from as far south as Esperence and north to Shark Bay. They inhabit deep reefs usually in waters greater than 30 metres (Fisheries dept. WA 1993). They move into shallower water twice a year in March and again in September (F.Van der Pol, Ledge Point, WA, pers comm 1996)) and can be caught in as little as 5 metres. WA Dhufish are slow growing, reaching sexual maturity at four to five years of age (Fisheries dept. WA 1993). They can grow to sizes greater than 25 kg.

The increasing numbers of amateur boats licensed each year combined with the advances in fishing technology have dramatically increased the pressure on this species. Good management is required to ensure we do not place the future of this species in jeopardy. Current management of the WA Dhufish is based on a bag limit (4 per day) for amateur fishermen and on the release of undersized fish (minimum legal size - 50cm), which applies for all fishermen.   However, this management can only be effective if fish that are released survive. The returning of undersized Dhufish will be of no service to the conservation of the fishery if the fish die from the effects of capture and handling.

The Aquaculture Development Unit is currently researching into the spawning and culture of the Westralian Dhufish. In the time they have been collecting fish from the wild for broodstock, a general trend has been observed that fish caught in over 20 metres of water do not survive back in the hatchery tanks. They either die shortly after capture or within a few days or weeks after relocation to the hatchery. Fish caught under 20 metres adapt well to the change and survive in the hatchery (G. Jenkins, Aquaculture Development Unit, South Metropolitan TAFE, pers comm 1996).

It is common knowledge within the fishing industry that considerable damage is sustained by fish caught from great depth. Common damage seen includes protrusion of the eyes, over-inflation and often bursting of the swim bladder, inversion of the stomach out of the mouth and displacement of internal organs through the anus. Haemorrhaging and gas bubbles are often seen throughout the organs of the fish. Assuming that the fish is in good health before being caught, significant damage and mortalities could be due to the rapid depressurisation from depth. The physics of inert gas bubble formation upon rapid and extensive reduction in barometric pressure apply to all living things. The effects on Dhufish would be analogous to those seen in decompression sickness in divers. This report describes an experiment to test the hypotheses that upon capture, Westralian Dhufish sustain damage caused by decompression sickness and that damage sustained by Westralian Dhufish increases with increasing depth.

2. Literature Review

2.1. The Solubility of Gases in the Ocean

All of the atmospheric gases are found in solution in sea-water (Sverdrup *et al* 1942). The concentration of these dissolved gases depends on the partial pressure of that gas in the atmosphere and its solubility (Henry's law). Solubility increases with molecular weight and decreases with increasing temperature and salinity.

In studies of the distribution of dissolved gases in the sea it is generally assumed that whatever the location of a water particle, at some time it has been at the surface and in equilibrium with the atmosphere. So when we examine deep sea water we would expect the gas content, except for oxygen and carbon dioxide, to be the same as the expected gas content at the surface. Oxygen and carbon dioxide concentrations would be different as they are consumed biologically. In studies of dissolved nitrogen (N_2), it has been found that sea water is virtually saturated (referred to a normal atmosphere) regardless of depth, making this assumption valid (Rakestraw & Emmel 1937, Broecker 1974, Shilling *et al* 1976, Saunders 1953). This also indicates that biological activity involving either fixation or production of N_2 is not sufficient to affect significantly the concentration of this gas in the water (Rakestraw & Emmel 1937).

Depending on the temperature of the surface water and with a normal ocean salinity of 35 parts per thousand (ppt), the concentration of dissolved nitrogen normally ranges between 8.4 and 14.5 ppt (Sverdrup *et al* 1942). Nitrogen is present at a partial pressure of 0.79 atm at all depths in ocean water in all but exceptional circumstances (Saunders 1953, Shilling *et al* 1976). Nitrogen represents the major inert gas in sea water, with other inert gases (Argon, helium, etc.) combining to equal only 2.7% of the dissolved nitrogen (Harvey 1955).

2.2. The Physics of the Ocean and Gases.

Gases are highly compressible due to the random free nature of their particles. The pressure of a given volume of gas, where temperature remains unchanged, varies inversely to its volume according to Boyle's law:

$PV = K$ where P = pressure

V = volume

K = constant

If the pressure of a given amount of gas is doubled, it must be compressed to half its volume. A balloon filled with a given amount of gas can be used to illustrate the effects of increasing pressure in the underwater environment (Figure A1). At the ocean surface the balloon is subjected to the atmospheric pressure at sea level. This is the pressure of air directly above the balloon taken to be 760mm Hg or 1 atmosphere (1atm), disregarding any daily fluctuations. Pressure increases by

1atm every 10 metres of water (Shilling *et al* 1976). Thus, when the balloon is at 10 metres in depth it is under 2atm of pressure. The doubling of the pressure at 10 metres means the balloon contracts to half its original volume. At 20 metres in depth the balloon is under a pressure of 3atm which causes the balloon to decrease to a third of its original volume at the surface and so on.

1 vol		1 atm	
	◯		surface
1/2 vol	◯	2 atm	10 metres
1/3 vol	◯	3 atm	20 metres
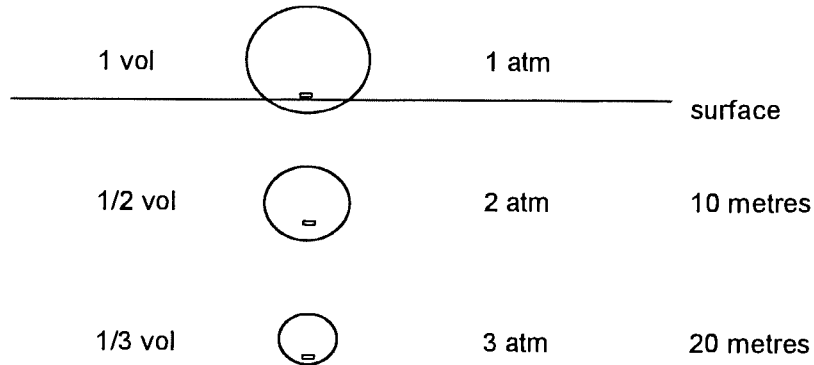
Figure A1: **The effects on the volume of a balloon as pressure increases with depth (according to Boyle's Law).**

The balloon can be taken as representing the air in the lungs of a diver. However, the lungs of a diver would crush under the increasing ambient water pressure. The air inside the lungs must be kept at a constant volume with increasing ambient pressure. To keep a constant volume, the air inside the lungs must be kept at the same pressure as the ambient water pressure. This is achieved by pumping more air into the lungs as the diver moves deeper and is subjected to increasing pressure. This is illustrated in Figure A2 using the balloon example again. At 10 metres, 2atm of gas would need to be supplied to the balloon to keep its volume the same as it was at the surface, while at 20 metres, 3atm of gas would be required. The extra air required by the diver can be supplied from a source of compressed air at the surface or from gas cylinders carried by the diver (Shilling *et al* 1976).

1 vol	1 atm	1 atm	
			Surface
1 vol	2 atm	2 atm	10 metres
1 vol	3 atm	3 atm	20 metres
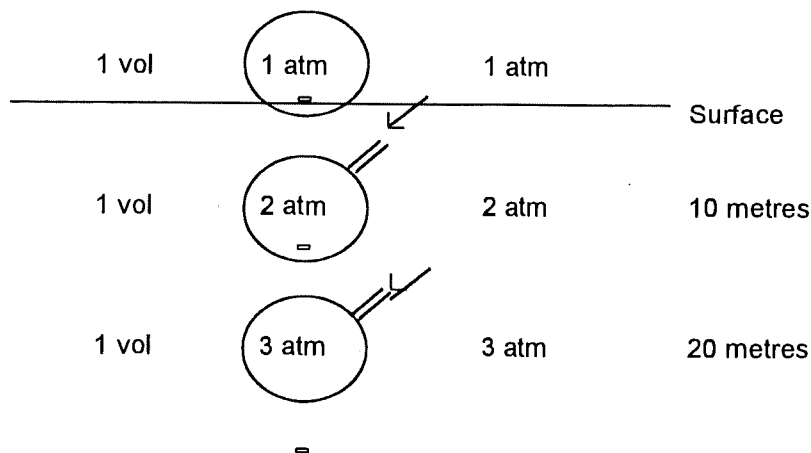
Figure A2: **To maintain a constant volume in the balloon, gas has to be pumped into it as pressure increases with depth.**

The balloon can also represent the swim bladder in a fish which must be kept at a constant volume to maintain neutral buoyancy. This is achieved by the secretion of gas in and out of the swim bladder as the ambient pressure changes. As a fish moves deeper, gas is secreted into the gas bladder from the bloodstream and when the fish moves up the water column, gas is absorbed back into the blood from the bladder.

2.3. Decompression Sickness in Humans

Decompression sickness, also referred to as the bends, caisson disease (in reference to tunnel workers), compressed air illness or dysbarism is a syndrome associated with a rapid and extensive reduction in environmental barometric pressure (Philp 1974). Not only has it been encountered by deep sea divers, but also by high altitude aviators and men employed in underground engineering projects in which compressed air is used to hold back ground water. It is also a potential hazard for astronauts and commercial airline passengers, should there be an accidental loss of cabin pressure (Philp 1974).

As the pressure of air increases inside the lungs of a diver to match the ambient water pressure, there is an increase in the partial pressures of the individual gas components of the air mixture being breathed in. Excluding CO_2 and other trace elements, air is made up of a mixture of the gases Nitrogen (79%) and Oxygen (21%) (Shilling et al 1976). Normal atmospheric pressure at sea level is 1atm or 760mm Hg. Thus the partial pressures of nitrogen (N_2) and oxygen (O_2) are:

$$P\ (N_2)\quad =\quad 79\%\ of\ 760mm\ Hg\quad =\quad 600mm\ Hg\ or\ 0.79atm$$

$$P\ (O_2)\quad =\quad 21\%\ of\ 760mm\ Hg\quad =\quad 160mm\ Hg\ or\ 0.21atm$$

As the diver descends, the pressure of air being supplied to the lungs increases resulting in a corresponding increase in the partial pressures of N_2 and O_2. At 10 metres the partial pressures of these gases doubles (1.58 atm and 0.42 atm respectively). The increase in partial pressures of these gases has several physiological implications for the diver. Problems can arise such as oxygen toxicity, nitrogen narcosis (a manifestation of general anaesthesia), high-pressure neurological syndrome (the symptoms caused by pressure alone) and decompression sickness (Smith 1984). It is the absorption of increasing amounts of nitrogen with increasing water pressure that leads to decompression sickness in humans. The focus of this study is on decompression sickness (also known as 'the bends') which is caused by the formation of inert nitrogen gas bubbles in the blood and tissues as a result of a sudden lowering of the ambient pressure. Nitrogen gas is chemically inert in that it is not significantly affected by biological processes (Sverdrup et al 1942). Oxygen gas does not cause decompression sickness unless at very high pressures as it is consumed biologically in metabolism and is bound and transported by haemoglobin (Beyer et al 1976, Shilling et al 1976). Other inert gases such as argon, neon and helium can play a role in decompression sickness, however, these gases are present in only minute quantities even at high partial pressures.

Study of decompression sickness has not advanced very far during this century. The early theories of Haldane and his associates (Boycott *et al* 1908) are still used today for the modelling of decompression schedules. Haldane postulated that during diving the body absorbs the excess nitrogen supplied to the lungs as a result of the increase in pressure and that this is the gas responsible for bubble formation during decompression (Boycott *et al* 1908). This nitrogen is distributed through the body via the blood. Nitrogen is absorbed by the various body tissues according to their relative solubility and the blood supply to that tissue (Shilling *et al* 1976). All the inert gases dissolve in blood and tissue according to Henry's law:

$C_x = a_x P_x$ where   C = dissolved concentration [cm^3 (STP)/cm^3 fluid]

a = absorption constant [cm^3 (STP)/cm^3 fluid atm]

P = partial pressure [atm]

x = inert gas

When a diver is exposed to an increased partial pressure of nitrogen, the body absorbs the excess gas through the lungs and into the bloodstream. Nitrogen dissolves into the body tissue and fluids and with time the dissolved concentration will reach equilibrium with the pressure in the lungs (Shilling *et al* 1976). After equilibrium is reached, no more gas dissolves. Thus if divers stay at a certain depth for long enough, they will absorb nitrogen to saturation. After this point is reached, there is no further decompression liability.

The solubility of inert gases is greater in fat tissue than in lean tissue as these gases have a greater affinity for lipoidal fluids (Shilling *et al* 1976). The relative solubility of nitrogen in different fluids can be seen in Table A1. Thus, fat tissue represents a large reservoir for dissolved nitrogen. Increasing age and body weight increases susceptibility to decompression sickness (Shilling *et al* 1976).

Table A1   Solubility coefficients for Nitrogen gas in biological fluids at 37°C (Shilling *et al* 1976).

	Blood	Lean Tissue	Fat Tissue	Olive Oil
Solubility of N_2	0.013	0.012	0.062	0.067

At equilibrium the dissolved N_2 is at a maximum and the body tissues are considered saturated. A decrease in absolute pressure results in a decrease in the maximum N_2 that can be dissolved in tissue according to Henry's law. The tissues become supersaturated and nitrogen must be removed to re-establish equilibrium. If the decompression is slow, the excess nitrogen can be removed through the blood to the lungs for exhalation (Shilling *et al* 1976). Rapid decompression

saturates the rate of nitrogen elimination leading to the dissolution of nitrogen from the blood and tissues. The nitrogen leaves solution into bubbles until the concentration of dissolved nitrogen re-establishes equilibrium with the reduced partial pressure in the lungs. This situation is analogous to the precipitation of solute from a supersaturated solution and is frequently compared to the formation of bubbles associated with the opening of a soft drink bottle. The formation of intravascular bubbles obstructs the blood stream slowing the blood flow to the lungs, further reducing the rate of nitrogen elimination (Mitchell 1995).

The earliest bubbles are intravascular with the number of bubbles and sites of formation dependant on the magnitude of the decompression (Daniels 1984). However, Smith (1984) found that the severity of bubble formation is only moderately receptive to pressure change and that even in the most severe decompressions the level of bubble formation appears to reach a limiting value. It is the accumulation of stationary bubbles in the bloodstream and tissues that lead to the symptoms of decompression sickness (Daniels 1984). There is a critical end point in the amount of nitrogen bubbles released, which if passed, leads to the symptoms of decompression sickness (Hills 1970, Buckles 1968). Thus, even in 'safe' dives there is still some bubble release, known as 'silent bubbles'. The incidence of decompression sickness with multiple dives is due to the accumulation of these 'silent bubbles'.

The Effect of Bubbles

Several haematological changes are associated with bubble formation in the blood due to the highly active gas-blood interface. Bubble pressure can lead to protein denaturation, similar to that caused by increased temperature (Smith 1984). Extravascular bubbles can rupture cell membranes releasing cell enzymes and lipids into the vascular system. Studies have shown that significant elevations in certain enzymes are indicative of tissue trauma and can be measured to gauge the extent of damage (Shilling *et al* 1976). The increase in circulating lipids leads to massive amounts of fat embolism, especially in the pulmonary capillaries and arterioles (Clay 1963). Intravascular bubbles can lead to air embolisation, blocking the flow of blood resulting in anoxic injury to the tissues (Shilling *et al* 1976, Weitkamp & Katz 1980). If bubble pressure is great enough, the vessel can rupture resulting in the haemorrhaging of blood into body tissue and the formation of clots at the damaged site (Thomson 1967, Kulshrestha & Mandal 1982). With increasing bubble formation, there is an associated increase in haemorrhaging and clotting.

2.4. Decompression Sickness in Fish

Decompression sickness in man is a result of basic physics which apply to all things in nature. It is reasonable to say that the effects of decompression would be similar to all living organisms. The capture of a fish involves a rapid retrieval from depth and is generally associated with a huge pressure reduction. The effects of a rapid decompression in a fish would be analogous to those found in man. The swim bladder in a fish is similar to the lungs of a diver in structure, however, its

function is to keep the fish neutrally buoyant. When a diver ascends from depth, the expanding gas in the lungs from the reduction in pressure (according to Boyle's Law), can be easily expelled through exhalation. Some fish, known as physostomes have a swim bladder that is connected by an pneumatic duct to the gut which allows the fish to expel gas directly from the swim bladder upon pressure reduction (Saunders 1953, Ferguson 1989). Westralian Dhufish are physoclists, which have a closed swim bladder, lacking a pneumatic duct (Saunders 1953). There is a well-developed capillary mesh that supplies blood to the swimbladder (Ferguson 1989) and this is the means by which gas enters and leaves the swimbladder. Therefore, upon pressure reduction, the expanding gas in the swim bladder must be removed via the bloodstream (Saunders 1953). If the pressure reduction is rapid, the gas entering the bloodstream is under pressure and the blood quickly becomes supersaturated. This leads to the formation of bubbles in the blood and the effects of decompression sickness. At the same time, dissolved nitrogen in the body tissues is coming out of solution and forming more bubbles, compounding the damage. Fish are subjected to both forms of bubble formation, while divers are only subjected to the formation of bubbles from dissolved nitrogen in the body, as they can expel expanding air in their lungs.

The expansion of gas according to Boyle's Law, is enhanced by an increase in temperature. The temperature in the first 100 metres of the ocean does not change to any appreciable extent (~1° C) (Broecker 1974). However, the temperature of the air in summer (when most recreational fishing is done) would be higher than that of the water from which the fish was caught. This increase in temperature would enhance the expansion of the swim bladder and the formation of bubbles, making the damage worse.

D'Aoust and Smith (1974) suggested that fish might be more sensitive to decompression stress than higher vertebrates because of their poorly perfused white muscle. The diffusion limited nature of their white muscle means dissolved gas takes longer to be expelled from the body.

Few studies have been conducted on the effects of decompression on fish, most of them in the USA. Feathers & Knable (1983) studied the effects of rapid decompression upon Largemouth Bass (Micropterus salmoides). They simulated the depressurisation from depth using a hyperbaric chamber in which the fish were acclimatised to the pressure of a certain depth and then subjected to rapid decompression which simulated conditions common to deep water angling. Fish depressurised from all depths experienced some bloating and external haemorrhaging. Fish decompressed from depths over 18.3 metres experienced severe internal haemorrhaging and formation of gas bubbles in the blood. These fish, subsequently had high mortality (at least 40%) as a result of decompression alone. The percentage of total mortality was directly related with the magnitude of decompression.

The damage found by Feathers and Knable (1983) included haemorrhaging in small blood vessels of the peritoneum, swim bladder, digestive tract, kidneys and those extending from the dorsal aorta. Large gas bubbles were found in the cardinal and hepatic veins, heart, gills and brain. While some bubbles could be tolerated, large gas bubbles in vital areas such as the heart were major

contributors to mortality. Fish depressurised from shallower depths exhibited bubbles, but not enough to cause significant mortality. These results agree with the observations made at the Fremantle Maritime College (G. Jenkins, Aquaculture Development Unit, South Metropolitan TAFE, 1996) in their collections of broodstock.

Beyer *et al* (1976) studied the effects of decompression induced bubble formation in salmonoids. They found that as the severity of the decompression increased, the number of bubbles found in the tissue increased. Post-mortem examination established that bubbles occluded blood vessels and were found throughout the heart. The coronary arteries were often completely occluded with bubbles, thus shutting off the blood supply to the heart. Blood vessels in the liver, kidney, brain, gonad and spleen also contained visible bubbles. If fish showed any signs of decompression sickness they rarely recovered.

Beyer *et al* (1976) also found that the time period between onset of signs of stress and death was considerably longer for large fish compared to small fish. Obvious bubbles were usually found in the fins, tail and lateral line in the small fish, but were generally lacking in the larger fish. They attributed this finding to the relationship between lethal bubble size and critical vessel size. Theoretically, similar ranges of bubble size would occur in both small and large fish. However, considering the embolic effects, bubbles of the same size would have a more severe effect on smaller fish due to their smaller vessel size, whereas larger bubbles or an accumulation of small bubbles would be required to cause ill effects in larger fish (Beyer *et al* 1976). This disagrees with the work by Feathers & Knable (1983) who found no significant difference in the damage between small and large fish. Considering that nitrogen is highly soluble in fat, one would expect to find a much larger reservoir of dissolved nitrogen in larger fish. The release of nitrogen could be expected to be proportional to the size of the fish. In studies of decompression sickness in man, increasing age and body weight increased the susceptibility to the disease (Shilling *et al* 1976). These findings also disagree with the observations made at the Fremantle Maritime College (G. Jenkins pers comm) that small fish have lower mortality and show a faster recovery from the capture.

Casillas *et al* (1975) investigated changes in haemostatic parameters in fish following rapid decompression. They found that decompression-induced intravascular bubbles caused mechanical damage to the tissue, which activated the haemostatic mechanism of the fish. This is also supported by the findings of Speare (1991). Coagulation or clotting occurs at the damaged site as a result. An activated coagulation system was found to enhance the pathogenesis of decompression sickness, often increasing the probability of death. More severe decompressions had higher levels of coagulation, increasing the rates of mortality.

2.5. Relationship to Gas Bubble Disease

Gas bubble disease (GBD) in fish is often compared to decompression sickness. They both involve the formation of gas bubbles by supersaturation of the body tissues with dissolved nitrogen. Activation of clotting mechanisms is evident in both diseases (Speare 1991). Intravascular bubble

formation and damage caused by bubbles to the tissue, including occlusion of vessels and anoxic injury are similar between the two diseases (Speare 1991). In GBD, the gradient of supersaturation is in the opposite direction to that of decompression sickness, in that supersaturated water gradually supersaturates the fish, whereas in decompression sickness, the body tissues absorb inert gas at the ambient hydrostatic pressure and then become supersaturated when the external pressure is decreased (D'Aoust and Smith 1974). The pathology of GBD differs in that it involves a condition of chronic exposure to low level super saturation (D'Aoust and Smith 1974), whereas, in decompression sickness, the pressure differences are greater and occur over a relatively shorter duration (Strauss 1979).

3. Materials and Methods

3.1. Experimental Procedure

Thirty WA Dhufish were caught from a boat using hand lines, over a period of five weeks between August and September. The fish were caught between 5 and 50 kilometres off Ledge Point which is approximately 140 kilometres north of Perth. 15 fish were caught in water less than 20 metres (shallow category) and 15 in water deeper than 20 metres (deep category). The minimum depth of capture was 9 metres up to a maximum depth of 73 metres. As WA Dhufish are a bottom dwelling species, depth of capture was recorded using the depth of the sea floor reading on an echo sounder. The fish were hauled to the surface as quickly as possible to simulate ordinary fishing technique (fisherman want to get the fish on board as quickly as possible). Length (tip of mouth to tip of tail) and weight of each fish were recorded upon capture.

The fish were killed as soon as practical and within five minutes after capture using cervical dislocation (spiking through the brain) as the method of euthanasia. The fish were immediately necropsied aboard the boat for examination of gross pathology. A visual examination of the skin, eyes, fins, opercula and gills was conducted and a damage score awarded. Scoring was assigned based on grading the degree of haemorrhaging and/or bubble formation. A score of 0 was awarded for no sign of damage. A score of 1, 2 or 3 was awarded for light, moderate or severe damage respectively. The gut cavity was opened and the degree of haemorrhage and/or bubble formation assessed for the gut flaps, liver, spleen, heart, kidney and swim bladder. Scores were assigned as before. Swim bladder inflation was based on a score of 1, 2 or 3. If the gut cavity was firm but not swollen, a score of 1 was awarded. If the gut cavity was swollen, a score of 2 was awarded. If the cavity was soft and upon dissection a rupture (large tear near the posterior end of swim bladder) was seen in the swim bladder, a score of 3 was awarded. The presence of exophthalmia was also scored. A score of 1 was awarded for its presence and a score of 0 if it was absent.

Small samples (~ 5mm x 5mm x 5mm) of the liver, spleen, heart and kidney were cut out and fixed in 10% formalin-seawater solution. These samples were taken back to the laboratory for subsequent histopathology examination. The tissues were dehydrated in an alcohol series,

embedded in wax and sectioned at 3 microns using standard techniques. Sections were routinely stained with Haematoxylin and Eosin. Slides were examined with a light microscope at 4x and 10x magnification, looking for acute damage in the various tissues. Acute damage was distinguishable by limited response to damage by the tissue (eg. Granule formation, white blood cell accumulation). Damage that was assessed included the formation of bubbles, blood clotting and haemorrhaging.

The presence of bubbles inside vessels, in blood and the tissue itself was given a score from 0-3. If no bubbles were present a score of zero was awarded. The presence of small bubbles only (did not displace tissue to any degree) was awarded a score of 1. The presence of small bubbles with a small number of large bubbles (displace tissue) and/or partial occlusion of vessels by bubbles was awarded a score of 2. Many large bubbles and the complete occlusion of vessels with bubbles was awarded a score of 3.

Clotting was distinguishable by pink areas in the form of a fibrous protein matrix with small amounts of white blood cell accumulation. Haemorrhaging was identified by the presence of blood through the tissue with no form of vessel or wall separating them. A scoring system of 0-3 was also used to grade the degree of clotting and haemorrhaging. A score of 0 was awarded for no clotting or haemorrhaging. If the clotting or haemorrhaging was greater than 0% but less than 5% of the tissue, a score of 1 was awarded. If the clotting or haemorrhaging was between 5% and 10% of the tissue, a score of 2 was awarded. A score of 3 was awarded if the clotting or haemorrhaging covered more than 10% of the tissue.

The presence of other indicators of tissue damage were recorded such as oedema and vessel breakage's, however, these were not scored. Oedema was identified by the swelling of intercellular spaces with fluid from broken blood or lymph vessels.

3.2. Statistical Analysis

The construction of box plots illustrated that the data was not normally distributed. Mann-Whitney U tests (Zar 1984) which test non-parametric data were employed to compare the two depth categories for the different parameters measured. All statistical tests were performed at the $P = 0.05$ level.

4. Results

Haemorrhaging, clotting and bubbles were present in all of the fish studied in both the gross and histopathology examinations. The mean damage scores for the deep water fish were all higher than the mean scores for the shallow fish (except kidney clotting).

4.1. Gross Pathology

After capture, all fish showed signs of bubble formation in the fins, opercula and gut flaps (Fig A4, Table A2). However, mean scores were quite low (mean score <1.5) and there was no significant

difference between depth categories. Large bubbles could be seen in the lining at the base of the gills in some of the fish from deep water.

Table A2: Mean damage scores and standard errors for gross bubble formation in WA Dhufish upon capture from two depth categories. (Scores: 0-3)

Depth Category	Fin bubbles	Opercula bubbles	Gut flap bubbles
Shallow (< 20m)	0.3 ± 0.11	0.2 ± 0.11	0.6 ± 0.15
Deep (> 20m)	0.3 ± 0.13	0.7 ± 0.24	1.4 ± 0.26
P-value	0.317	0.637	0.157

Haemorrhaging was evident in the liver (Figs A5 and A6), heart and swim bladder, especially for the deep water fish with mean scores of 2.4 ± 0.12, 1.5 ± 0.32 and 1.7 ± 0.28 respectively (Table A3). The haemorrhage scores for the deep water fish were all significantly higher than the scores for the shallow water fish. Swim bladder haemorrhaging occurred in the network of capillaries on the inside lining of the swim bladder. The difference in haemorrhage severity between shallow and deep water fish is illustrated in Figures A7 and A8.

Gross haemorrhaging could not be distinguished in the kidney or spleen due to the dark colour of these organs. Evidence of bubbles was also difficult to distinguish in the organs of the fish. However, bubbles were present in the gut lining and in the residue blood on the surface of the organs. The presence of bubbles in these areas indicates that gas was escaping.

Table A3: Mean damage scores and standard errors for gross haemorrhaging in WA Dhufish upon capture from two depth categories. (Scores: 0-3)

Depth category	Liver haemorrhage	Heart haemorrhage	Swim Bladder haemorrhage
Shallow (< 20m)	0.7 ± 0.18	0.5 ± 0.17	0.3 ± 0.19
Deep (> 20m)	2.4 ± 0.12	1.5 ± 0.32	1.7 ± 0.28
P-value	0.001	0.014	0.003

All fish displayed some degree of swim bladder inflation (Fig A9, Table A4), even if it was just normal inflation (score of 1). 12 out of the 15 fish in the deep water category ruptured their swim bladder upon capture (mean score of 2.8 ± 0.11). None of the shallow water fish ruptured their swim bladder (mean score of 1.2 ± 0.10). The scores for inflation of the swim bladder for deep fish were higher than the scores for shallow fish. However, the difference just failed to reach significance (P=0.083)

Figure A10 shows a deep water fish with protrusion of the eyes or exophthalmia. This was seen in 7 out of the 15 deep water fish compared to no presence of exophthalmia in the shallow water fish (Table A4). Severe exophthalmia and bubble formation inside the eyes was evident in one of the smaller deep water fish.

Table A4:   Mean swim bladder inflation scores and presence of exophthalmia in WA Dhufish upon capture from two depth categories (with standard errors)

Depth category	Exophthalmia (0=absent, 1=present)	Swim Bladder inflation (score: 1-3)
Shallow (< 20m)	0.0	1.2 ± 0.10
Deep (> 20m)	0.5 ± 0.13	2.8 ± 0.11
P-value	0.002	0.083

None of the symptoms noted in the gross pathology had any relationship with the size of the fish. There was no relationship between size of fish and depth of capture.

4.2. Histopathology

Heart

Bubbles and blood clotting were evident in the histopathology examination of the heart for almost all of the fish (Table A5, Figs A11 and A12). Deep water fish had a mean bubble score of 1.8 ± 0.19 compared to a score of 1.2 ± 0.14 for the shallow fish. The difference between bubble scores was significant (P = 0.050). Both depth categories had high levels of blood clotting in the heart, with a mean clotting score of 2.0 ± 0.19 for the deep fish and a score of 1.6 ± 0.24 for the shallow fish. There was no significant difference between clotting scores for the different depth categories. Haemorrhaging is not found in the heart as this organ does not have its own circulatory system in WA Dhufish, therefore, there are no vessels which can be ruptured.

Table A5   Mean damage scores and standard errors for heart histopathology in WA Dhufish upon capture from two depth categories. (Score: 0-3)

Depth category	Heart bubbles	Heart clotting
Shallow (< 20m)	1.2 ± 0.14	1.6 ± 0.24
Deep (> 20m)	1.8 ± 0.19	2.0 ± 0.19
P-value	0.050	1.000

Liver

Bubbles, clotting and haemorrhaging were all evident in the tissue of the liver (Table A6). Bubbles were very pronounced in all fish, occurring inside blood vessels and through the pancreatic tissue surrounding the vessels (Figs A13 and A14). Small bubbles were also present in the parenchyma. Mean bubble scores for deep and shallow fish were 2.1 ± 0.18 and 1.4 ± 0.20 respectively. There was no significant difference between depth categories for bubble scores.

Clotting could be seen in and around blood vessels throughout the liver in all of the fish (Fig A13). The level of clotting was significantly higher (mean score of 2.4 ± 0.18) in the deep fish than the shallow fish (mean score of 1.1 ± 0.18). Liver haemorrhage (Fig A15) was not as pronounced as other effects with scores for deep and shallow fish of 1.1 ± 0.19 and 0.9 ± 0.25 respectively. The difference in haemorrhage scores for the two depth categories was not significant.

Other notable observations in the liver histology were the presence of empty blood vessels and large gaps between the vessels and the parenchyma tissue (Fig A16). Several empty broken vessels were noted, usually with heavy clotting through the damaged areas.

Table A6: Mean damage scores and standard errors for liver histopathology in WA Dhufish upon capture from two depth categories. (Score: 0-3)

Depth category	Liver bubbles	Liver clotting	Liver haemorrhage
Shallow (< 20m)	1.4 ± 0.20	1.1 ± 0.18	0.9 ± 0.25
Deep (> 20m)	2.1 ± 0.18	2.4 ± 0.18	1.1 ± 0.19
P-value	0.564	0.005	0.317

Kidney

Bubbles, clotting and haemorrhaging were all seen in the kidney (Table A7). High mean scores of 2.6 ± 0.13 for deep fish and 1.9 ± 0.21 for shallow fish were recorded for bubbles. Bubbles were usually small and associated with oedema. Occlusion of blood vessels with bubbles were often seen. Some large bubbles were associated with areas of haemorrhaging (Fig A17). The difference between bubble scores for the two depths was significant at a 90% confidence level (P = 0.105).

Mean clotting scores were approximately the same for both depth categories with no significant difference found. Clotting was often associated with bubbles inside blood vessels. Haemorrhaging was seen throughout the kidney tissue in the deep fish (mean score of 1.9 ± 0.22) as illustrated in

Figure A17. The deep fish scores were significantly higher than the haemorrhage scores for the shallow fish (mean score of 0.4 ± 0.22).

Vessel wall damage was evident in the kidney in both depth categories. The large spaces that were present between the vessels and parenchyma in the liver were not present in the kidney.

Table A7:   Mean damage scores and standard errors for kidney histopathology in WA Dhufish upon capture from two depth categories. (Score: 0-3)

Depth category	Kidney bubbles	Kidney clotting	Kidney haemorrhage
Shallow (< 20m)	1.9 ± 0.21	1.6 ± 0.29	0.4 ± 0.22
Deep (> 20m)	2.6 ± 0.13	1.5 ± 0.25	1.9 ± 0.22
P-value	0.105	0.778	0.003

Spleen

Table A8 shows that bubbles and clotting were present in the spleen of most fish. Bubbles were mostly small and associated with oedema. Bubbles were also present inside blood vessels. The bubbles scores for deep and shallow fish (mean scores of 1.8 ± 0.19 and 1.4 ± 0.15 respectively) were not significantly different.

Table A8:   Mean damage scores and standard errors for spleen histopathology in WA Dhufish upon capture from two depth categories. (Score: 0-3)

Depth category	Spleen bubbles	Spleen clotting
Shallow (< 20m)	1.4 ± 0.15	0.5 ± 0.17
Deep (> 20m)	1.8 ± 0.19	0.9 ± 0.28
P-value	0.450	0.599

Clotting in the spleen (Fig A18) was only present in half of the fish, giving low mean clotting scores for both deep and shallow fish (0.9 ± 0.28 and 0.5 ± 0.17 respectively), with no significant difference between depths. Clotting was often associated with bubble formation inside the blood vessels. Haemorrhaging could not be distinguished in the spleen tissue, however, some damage to vessel walls was evident.

None of the symptoms noted in the histopathology examination had any relationship with the size of the fish.

5.   Discussion

The most important findings of this study was that there was the presence of bubbles, clotting and haemorrhaging in all fish in both the gross and histopathology examinations. This support the first hypothesis that the Westralian Dhufish suffers damage caused by decompression sickness upon capture. The presence of bubbles is the most important factor in determining the presence of decompression sickness as they are formed as a direct result of the decrease in barometric pressure (Shilling *et al* 1976, Mitchell 1995, Daniels 1984, Boycott *et al* 1908, Smith 1984). In this experiment, bubbles found in the gross examination also corresponded to the presence of bubbles throughout all the major organs in the histopathology examination. Clotting, haemorrhaging and vessel damages are directly related to the pressure of bubbles (Shilling *et al* 1976, Smith 1984). The presence of these forms of damage in both examinations gives further support to the hypothesis

The results indicate that the damage in the deep water fish is greater than the shallow water fish which supports the second hypothesis that damage is greater in fish caught from deep water. With increasing level of bubble formation, there is an associated increase in the other forms of damage (i.e clotting and haemorrhaging). This agrees with the results of Feathers and Knable (1983), Beyer *et al* (1976) and Casillas *et al* (1975). Therefore, increases in the level of bubble formation and other associated damage is related to the extent of decompression.

5.1. Gross Pathology

The gross examination of the heart, liver and swim bladder showed that haemorrhaging was significantly higher in the deep water fish. This supports the second hypothesis that damage is greater in fish caught from deep water. However, bubble formation in the fins, opercula and gut flaps did not show significant differences between depth categories. This could be explained by the fact that the gross bubble scores were fairly conservative estimates and yielded a low result. External bubbles in the tissue are very small and invisible to the naked eye. They could be felt popping under the pressure of a finger, however, the severity of formation was too difficult to determine. Combined with this is the short life span of these external bubbles as they disappear quite quickly.

The formation of large gas bubbles in the residue blood in the gut cavity (Plate.2) and the inflation of the lining of the gut and at the base of the gills indicated that gas was being released. Even though this was not associated with any particular organ, it indicates the presence of decompression sickness, therefore, supporting the first hypothesis.

Despite the difference in swim bladder inflation for the two depths not reaching significance (P=0.083), 12 out of the 15 fish caught in deep water ruptured their swim bladder, while none of the shallow water fish did. As with all non-parametric statistical analysis, the Mann-Whitney U test is very conservative in predicting a significant difference in results and could explain the non-

significance of the results. However, there was a strong difference in swim bladder inflation between depths and this presents some scope for further research. Exophthalmia, which results from the formation of gas bubbles behind the eye, was present in half of the deep water fish with no evidence in the shallow water fish. This further supports the hypothesis that damage increases with depth.

5.2. Histopathology

Significant differences found between depth categories in the histopathology examination are supported by the findings of Feathers and Knable (1983) and Beyer et al (1976) and support the second hypothesis being tested. Tissues where the difference in damage scores between depths was not significant could be attributed to the nature of histopathology examination. Because such a thin section of tissue is examined, a damaged area is easily missed by the tissue sectioning process. The fact that not all the differences in damage scores between depths were significant does not reduce the importance of the results. The health of the fish depends on the condition of all its organs, so when the parameters are combined together, deep fish will sustain more damage than shallow fish upon capture.

A large proportion of the bubbles in the liver, kidney and spleen were contained in vessels (Fig A13). Daniels (1984) indicated that the earliest bubbles are intravascular. This is due to the large loads of dissolved nitrogen in the blood. With the reduction in barometric pressure, the body seeks to remove excess nitrogen through the gills via the blood (Shilling et al, 1976). Thus, there is huge 'dumping' of dissolved nitrogen from the tissues into the bloodstream. This leads to the occlusion of blood vessels with bubbles coming out of solution. These results are supported by the findings of Feathers and Knable (1983), Beyer et al (1976) and Casillas et al (1975).

Clotting in the blood vessels arises from the mechanical damage caused by bubbles inside the vessel (Casillas et al 1975, Speare 1991). Severe bubble pressure causes vessels to rupture resulting in mass clotting at the damaged sites. The empty blood vessels seen in the liver, kidney and spleen (Fig A16) are due to the breakage of the blood vessel somewhere along its length, resulting in the emptying of the blood from the vessel. The presence of empty vessels agrees with the findings of Kulshrestha and Mandal (1982) in their study of the pathology of gas bubble disease.

The large spaces that are present between the vessels and the parenchyma in the liver (Figs A13 and A16) can also be attributed to intravascular bubbles. The presence of the bubbles causes the vessel to expand, compressing the surrounding parenchyma tissue. When the blood vessel breaks somewhere along its length, the pressure is released and the vessel contracts back to its original form. However, the parenchyma does not return to its original position and a space is left (B.Jones, pers comm). This gap does not occur in the kidney or spleen as these tissues are quite spongy and return to their original shape when the pressure in the vessel is released.

5.3. Conclusion

In conclusion, both the hypotheses were supported by the results and are supported by other studies in this area. The damage sustained by the fish is analogous to that found in human decompression sickness and is in agreement with the principles of depressurisation. Management implications cannot be drawn as a result of this study alone, however, if further research indicates that the released fish do not survive as a result of decompression sickness, then new management strategies will need to be found.

A smaller bag limit for recreational fishermen seems to be one of the obvious management options for the WA Dhufish. The current limit of 4 per day is far too high considering the size of these fish. The removal of minimum size limits seems an easy solution to the problem of decompression induced mortalities of released fish. However, fishermen are likely to throw back small Dhufish that are already dead when they land bigger fish. The splitting of the bag limit into a small fish category and a large fish category is also a possible solution to prevent fishermen throwing back dead fish. With increasing pressure on Dhufish stocks, management needs to be reassessed to ensure the conservation of this fishing species.

5.4. Scope for Further Research

Further research is required to test the long term survival of WA Dhufish after being caught. Treatment of divers bends involves recompression back to the pressure they were diving at. If fish are quickly returned to depth, can the damage be reduced and will the fish survive? Tagging studies will need to be employed to study the survival of released fish.

Studies by Hills (1970) and Buckles (1968) showed that there was a critical level of bubbles, which if passed would result in death. Is their a critical depth at which fish can be caught and safely returned based on a maximum level of damage that the fish can tolerate?

This study found no relationship between size of fish and the degree of damage, nor was their any correlation between fish size and depth of capture. This lack of relationship agrees with findings by Feathers and Knable (1983), however, disagrees with the findings of Beyer et al (1976). It may be an artefact of the small sample size used in this experiment. To find a true relationship, further work must be conducted on long-term survival.

The fact that 12 of the 15 fish in deep water ruptured their swim bladder compared to no rupturing in the shallow water may pose a problem for management. A fish with a ruptured swim bladder would have little chance of survival if returned. It would not be able to remain neutrally buoyant and would sink to the bottom where it would be highly vulnerable to predation.
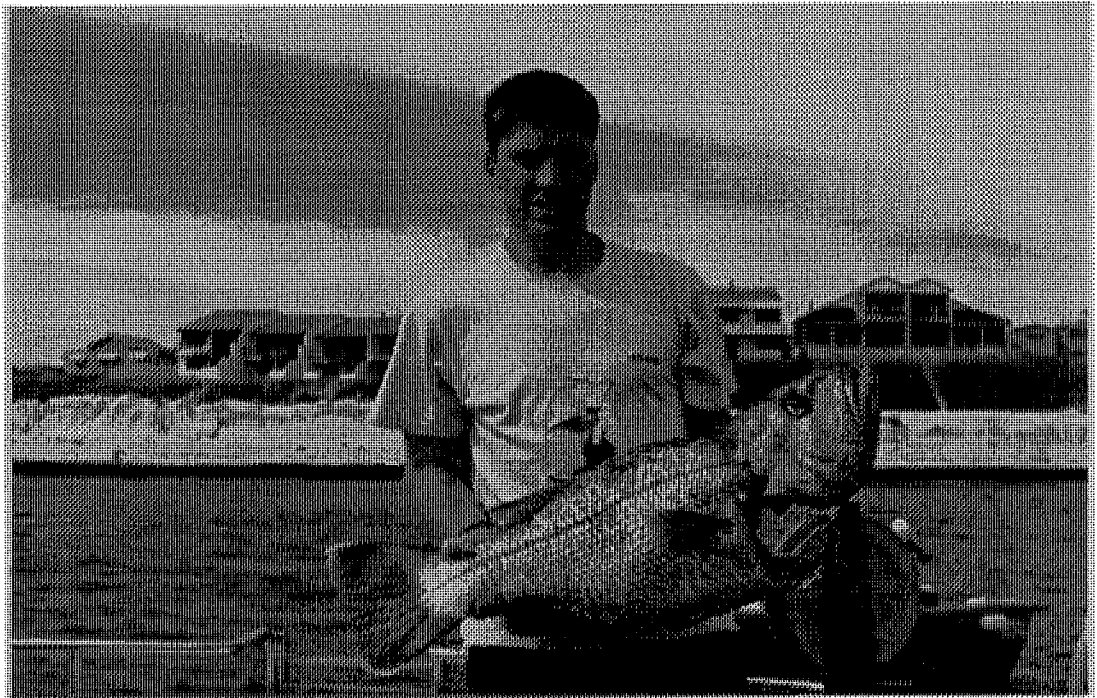
Figure A3: The Wetralian dhufish

Figure A4: The formulation of bubbles in the residue blood in the gut cavity

Figure A5: Shallow water fish with minor haemorrhage in the liver (red smear in yellow lobe at top of photo).

Figure A6: Deep water fish with moderate to severe haemorrhage in liver (red smearing through the yellow lobes).

Figure A7: Slight haemorrhaging in the capillaries on the inside of the swim bladder of a shallow water fish.

Figure A8: Severe haemorrhaging in the capillaries on the inside of the swim bladder of a deep water fish.

Figure A9: Heavy inflation of the swim bladder in shallow water fish
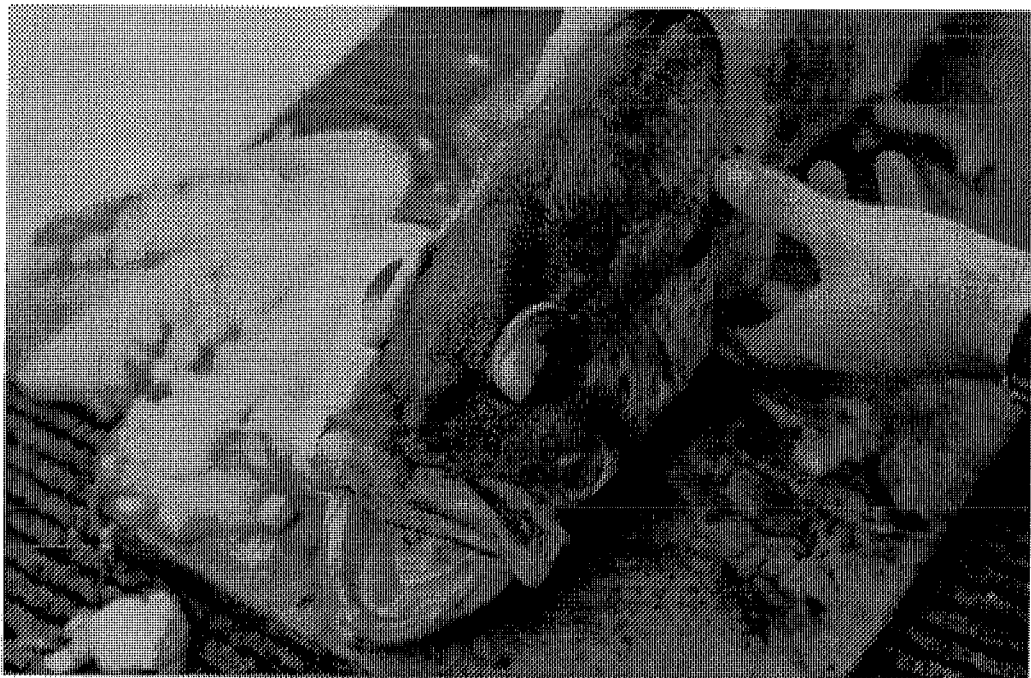
Figure A10: Exophthalmia (protrusion of the eyes) in a deep water fish.

Figure A11: (4x) Large bubbles in the tissue of the heart of a deep water fish.
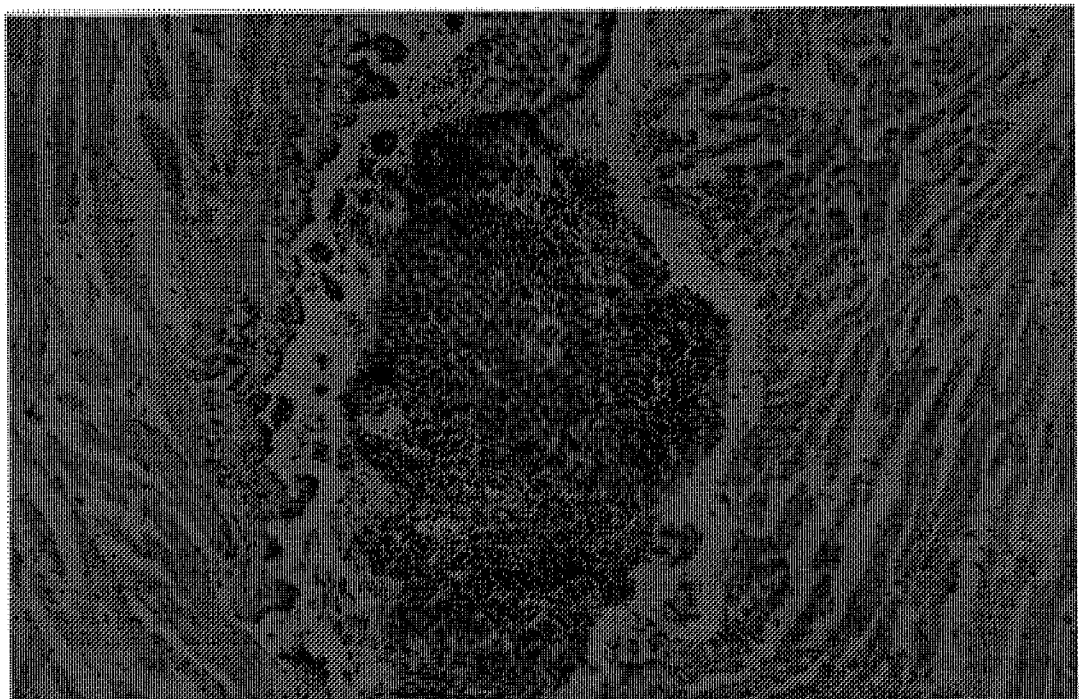
Figure A12: (10x) Heavy clotting in the heart tissue of a deep water fish.

Figure A13: (4x) Bubbles and clotting occluding a blood vessel in the liver of a
deep water fish.

Figure A14: (10x) Large bubbles in the pancreatic tissue (purple tissue)
surrounding a blood vessel in the liver.
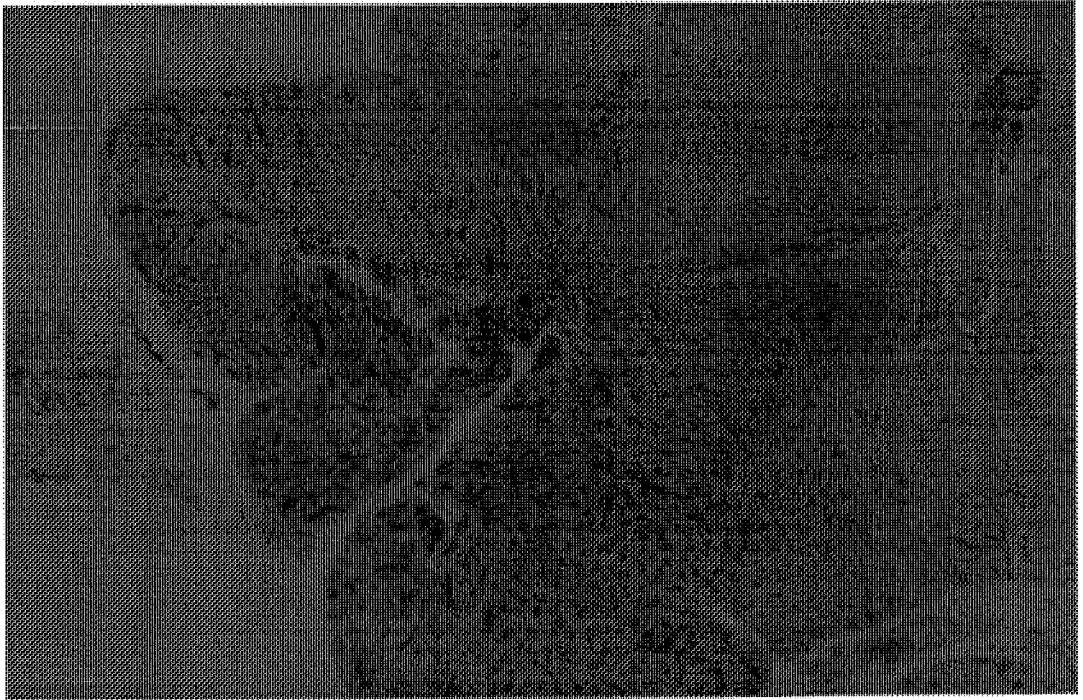
Figure A15: (4x) Severe haemorrhaging in the liver of a shallow water fish.

Figure A16: (4x) Empty blood vessels with large spaces between the vessel and the liver parenchyma.
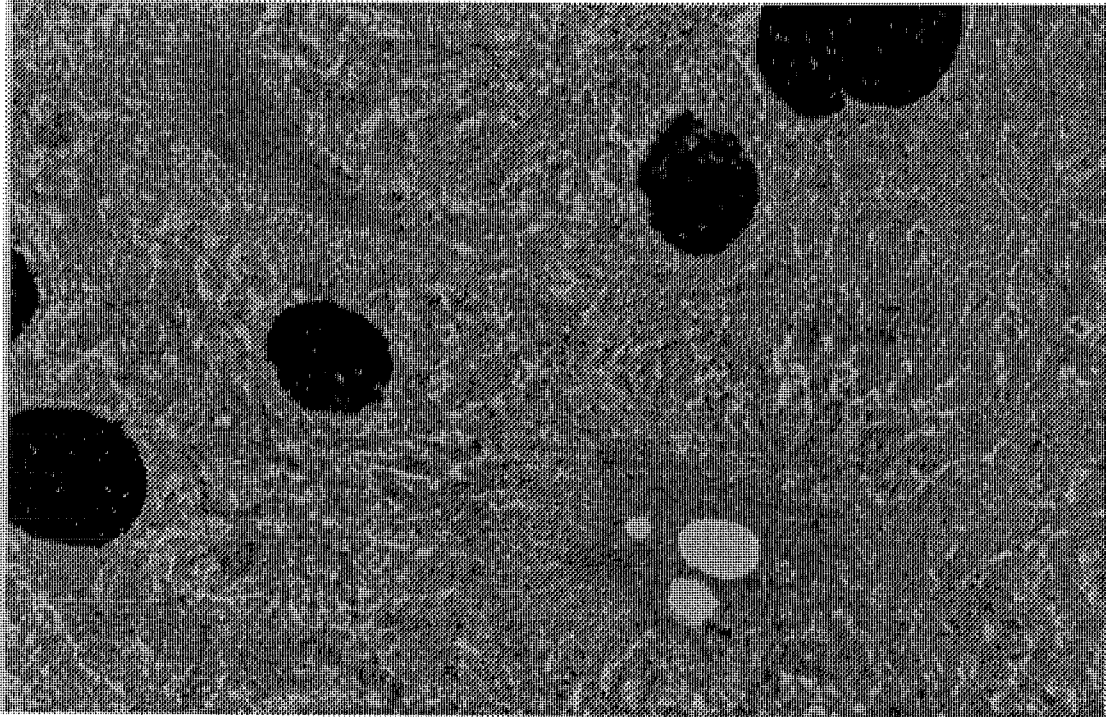
Figure A17: (10x) Haemorrhaging with large bubble formation in the tissue of the kidney.
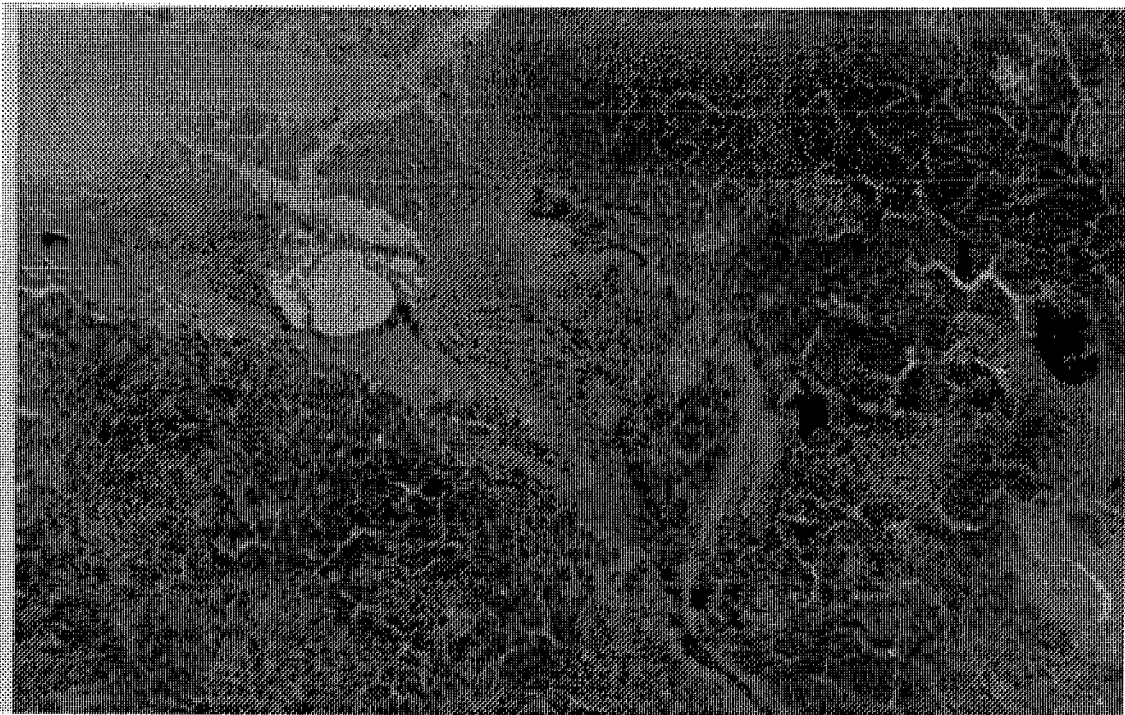
Figure A18: (10x) Heavy clotting in the spleen of a deep water fish (pink area in centre with white blood cell accumulation).

References

Beyer, D.L., D'Aoust, B.G. and Smith, L.S. (1976) Decompression-induced bubble formation in salmonoids: comparison to gas bubble disease. *Undersea Biomedical Research* **3 (4)**, 321-338.

Boycott, A.E., Damant, G.C.C. & Haldane, J.S. (1908) The prevention of compressed air illness. *J. Hyg.(London)* **8**, 342-443.

Broecker, W.S. (1974) *Chemical Oceanography.* Harcourt, Brace, Jovanovich, New York, 115-119.

Buckles, R.G. (1968) The physics of bubble formation and growth. *Aerospace Medicine* 1062-1069.

Casillas, E., Miller, S.E., Smith, L.S. and D'Aoust, B.G. (1975) Changes in hemostatic parameters in fish following rapid decompression. *Undersea Biomedical Research* **2 (4)**, 267-276.

Clay, J.R. (1963) Histopathology of experimental decompression sickness. *Aerospace Medicine* **34**, 1107-1110.

D'Aoust, B.G. and Smith, L.S. (1974) Bends in fish. *Comp. Biochem. Physiol.* **49A**, 311-321.

Daniels, S. (1984) Ultrasonic monitoring of decompression procedures. In: *Diving and Life at High Pressures.* The Royal Society, London, 153-170.

Feathers, M.G. and Knable, A.E. (1983) Effects of decompression upon Largemouth Bass. *North American Journal of Fisheries Management* **3**, 86-90.

Ferguson Wood, E.J. (1975) *The Living Ocean.* Croom Helm, London, 74.

Ferguson, H.W. (1989) *Systemic Pathology of Fish.* Iowa State University Press, Ames, Iowa.

Fisheries Department of Western Australia (1993) Fish for the future card No. 2

Harvey, H.W. (1955) *The Chemistry and Fertility of Sea Waters.* Cambridge University Press, London, 183-187.

Hills, B.A. (1970) Limited supersaturation versus phase equilibrium in predicting the occurrence of decompression sickness. *Clinical Science* **38**, 251.

Kulshrestha, A.K. and Mandal, P.K. (1982) Pathology of gas bubble disease in two air breathing catfishes (*Clarias batrachas* Linn. and *Heteropneustes fossilis* Bloch.) *Aquaculture*, **27**, 13-17.

Mitchell, S.J. (1995) The role of lignocaine in the treatment of decompression illness. A review of the literature. *SPUMS Journal* **25 (4)**, 182-194.

Philp, R.B. (1974) A review of blood changes associated with compression-decompression: relationship to decompression sickness. *Undersea Biomedical Research* **1 (2)**, 117-150.

Rakestraw, N.W. and Emmel (1937) The relation of dissolved oxygen to nitrogen in some Atlantic waters. *Journal of Marine Research* **1**, 207-216.

Riley, J.P. and Chester, R. (1971) *Introduction to Marine Chemistry.* Academic Press, London, 111-115.

Saunders, R.L. (1953) The swimbladder content of some freshwater fish with particular reference to the physostomes. *Canadian Journal of Zoology* **31 (6)**, 547-560.

Shilling, C.W., Werts, M.F. and Schandelmeier, N.R. (1976) *The Underwater Handbook*. Plenum Press, New York and London, 85-90, 467-505.

Smith, E.B. (1984) The biological effects of high pressures: Underlying principles. In: *Diving and Life at High Pressures*. The Royal Society, London, 5-16.

Speare, D.J. (1990) Histopathology and ultrastructure of ocular lesions associated with gas bubble disease in Salmonoids. *J. Comp. Path.*, **103**, 421-432.

Speare, D.J. (1991) Endothelial lesions associated with gas bubble disease in fish. *J. Comp. Path.*, **104**, 327-335.

Strauss, R.H. (1979) Diving Medicine. *American Review of Respiratory Disease*, **119**, 1001-1023.

Sverdrup, H.U., Johnson, M.W. and Fleming, R.H. (1942) *The Oceans: Their Physics, Chemistry and General Biology*. Prentice Hall, New York, 186-192.

Thomson, W.A.R. (1967) *Black's Medical Dictionary* 27th Ed. Adam and Charles Black, London.

Walton Smith, F.G. (1974) *Handbook of Marine Science Vol 1*. CRC Press, Ohio.

Weitkamp, D.E. and Katz, M. (1980) A review of dissolved gas supersaturation literature. *Transactions of the American Fisheries Society*, **109**, 659-702.

Zar, J.H. (1941) *Biostatistical Analysis* 3rd Ed. Prentice Hall, New Jersey, c1996.

Appendix B Suitability of bore salt-water and ocean water for rearing dhufish

At the ADU there are two sources of salt water available. Ocean-intake water is pumped directly from the ocean, while bore salt-water is pumped from 18m below ground approximately 10m from the shoreline. The quality of ocean-intake water is greatly affected by the conditions of the adjacent Swan River Estuary. Storms and heavy rainfall can cause high sediment loads, and decreased salinity. Maintenance of water quality is particularly a problem during the winter months, when storms and high winds are common in Perth. Maintenance of pumps and filters during these months is expensive and time-consuming. In contrast, bore salt-water is not affected by the Swan River and is clean. Little filtration is needed before use in the hatchery, making bore salt-water cheap and efficient to maintain. Bore salt-water chemistry, however, differs to ocean-intake water, with the main area of concern being its high carbon dioxide levels coupled with low pH.

Over the past two years, bore salt-water had been used increasingly at the ADU. All larviculture and juvenile grow-out of snapper, black bream, King George whiting and dhufish are now conducted in bore salt-water with excellent results. In February 1999, due to a need for extensive maintenance on the ocean-intake pumps and filters, all broodstock at the ADU were temporarily converted to bore salt-water. Initially, no ill effects of the bore salt-water were noted among any of the broodstock (including snapper, black bream, King George whiting, and dhufish). A management decision was therefore made to keep the bore salt-water on in an effort to maintain better water quality during the winter months and reduce maintenance costs and labour. Most of the broodstock at the ADU of various species (other than wild-caught dhufish) continued to appear to thrive. Dhufish broodstock feed rates declined however, and six months after the bore salt-water had been turned on, wild-caught dhufish broodstock began to deteriorate and more than twenty of the thirty two fish caught as broodstock that year died over a period of several months. All these fish had been caught in shallow water (<20m) and handled according to best practice at the ADU. Fish health experts at Fisheries WA were unable to identify a particular cause of death. All fish that died had not eaten for some time. The ocean water was turned back on in November 1999, solely for the wild-caught dhufish. The health of the surviving wild-caught fish improved and they began to feed more vigorously, however, few sexually matured and those that did were weak and unable to withstand the rigours of hormonal manipulation.

As it was not possible to compare the health and survival of dhufish held in bore salt-water and ocean-intake water concurrently, it is impossible to determine whether the bore salt-water was the cause of the mortalities or not. Observations on feeding and behaviour, however, strongly suggest a connection. Similar mortality rates have not been observed in hatchery-reared dhufish. This is likely to be due to the fact that hatchery-reared fish are generally more tolerant to stress than their wild-caught counterparts. Further support for the difference in tolerance to bore water between hatchery-reared and wild-caught fish is given by observations on a bore-water tank containing 20 hatchery-reared dhufish and 2 wild-caught males. Although hatchery-reared fish continued to feed and thrive, the two males gradually stopped feeding and lacked vigour. In a similar tank, stocked with similar fish but run on ocean intake water, the two wild-caught males ate substantially more than their bore-water counterparts at equal temperature. The bore-water tank was therefore turned to ocean water and feeding increased in the males.

Since bore water is likely to be widely used in land-based aquaculture ventures the question of the suitability of bore salt-water for the culture of dhufish and other species must be fully explored and techniques developed for making it suitable, such as properly removing CO_2 or other contaminants. Addressing these issues is now a high priority at the ADU.

Appendix C   Specifications for vitamin and mineral premix

The diet of captive dhufish is supplemented regularly with vitamins and minerals. The vitamins and minerals are ordered from Gloucester Park Rural Industries, Leeville NSW according to the specifications in Table A9. It is supplied in two parts: Part A contains the vitamins in a sodium bicarbonate base; Part B contains minerals. These are mixed in 1:1 ratio and then stored in an airtight container in a refridgerator until use. The premix is administered to wild-caught broodstock in a gelatine capsule. The capsules are filled with the premix (0.6g/capsules) and then inserted into the feed prior to feeding. Approximately one capsule is given per kilogram of fish. Capsules are fed 3 times a week for two weeks every month. For hatchery-reared broodstock and juveniles, the premix is incorporated in a moist pellet at an inclusion rate of 0.5%/kg. The vitamin-enriched pellet comprises about 20% of the total diet (Refer to Appendix D for recipe for the moist pellet).

Table A9:   Specifications for vitamin and mineral premix.

Supplement	Source Ingrediant	Amount of Source Ingredient (g/kg premix)	Amount of Active Ingrediant (unit/kg premix)	Amount of Active Ingrediant/Capsule (1 capsule = 0.6 g premix)
Part A: Vitamins				
A	Vitamin A/D3 (500/100)	2.2	$1,103 \times 10^6$ IU	662,251 IU
D			220×10^6 IU	132,450 IU
B1	Vitamin B 1 (100%)	2.8	2.8 g	1.7 mg
B2	Vitamin B 2 (80%)	4.6	3.7 g	2.2 mg
B3	Niacin (100%)	36.8	36.8 g	22.1 mg
B6	Vitamin B 6 (100%)	2.8	2.8 g	1.7 mg
B12	Vitamin B12 (1%)	0.4	0.004 g	0.002 mg
C	Vitamin C (90%)	184.5	166.0 g	99.6 mg
E	Vitamin E (50%)	73.6	36.8 g	22.1 mg
K	Hetrazeen K 3 (22.7%)	16.2	3.7 g	2.2 mg
Pantothenic acid	D-Calcium Pantothenate (100%)	9.2	9.2 g	5.5 mg
Folic acid	Folic (95%)	1.0	0.961 g	0.6 mg
Biotin	Biotin (2%)	9.2	0.183 g	0.1 mg
Inositol	Inositol (100%	92.0	92.0 g	55.2 mg
Astaxanthin	Astaxanthin (8%)	23.0	1.8 g	1.1 mg
Ethoxyquin	Ethoxyquin (66%)	41.9	27.621 g	16.6 mg
Base	Sodium Bicarbonate	36.8	36.8 g	22.1 mg
Part B: Minerals				
Choline	Choline Chloride (60%)	423.4	254.1g	152.4 mg
Iodine	Potassium Iodide (90/10) (68% I)	0.2	0.1 g	0.069 mg
Copper	Copper Sulphate (25%)	2.0	0.5 g	0.3 mg
Iron	Ferrous Sulphate (30%)	16.9	5.1 g	3.0 mg
Manganese	Manganese Sulphate (31%)	4.7	1.5 g	0.882 mg
Zinc	Zinc Sulphate Mono (31%)	47.1	14.6 g	8.8 mg
Selenium	Sodium Selenite (1%)	5.6	0.1 g	0.034 mg
Total		**1 kg**		

Appendix D   Recipe for the moist pellet

Moist pellets are fed to hatchery-reared dhufish as a vector for administering vitamin supplements and other oral treatments (after Glencross et al, 1999).

Ingredients are outlined in Table A10. Whole pilchards, prawns and squid are minced, before being combined with dry ingredients (vitamins/minerals, gluten and fishmeal). All ingredients are mixed thoroughly using a commercial mixer. Fish oil and water are then added to make dough. The amount of water is adjusted to ensure the dough is of the desired consistency. The dough is then covered and placed in the refrigerator overnight to allow it to rest (this makes it easier to pass through the pasta maker) and to chill the dough prior to pressing (to counteract the heat generated by the pasta maker). The dough is then transferred into a pasta maker fitted with an appropriate sized die (depending on the size of pellet being made). Pellets are then baked in the oven at 80°C until a slight crust is formed (approximately 2 hrs), before being cooled at room temperature. When cool, the pellets are placed in airtight containers and stored in the freezer for up to 1 month.

Table A10:   Ingredients for moist pellet. After Glencross et al, 1999

Ingredient	Inclusion (%)	g/kg
Vitamin/Mineral premix	0.50	5.00
Water	12.00	120.00
Fish oil	5.00	50.00
Wheat gluten	10.00	100.00
Fish meal	42.50	425.00
Fresh pilchards	10.00	100.00
Whole prawns	10.00	100.00
Whole squid	10.00	100.00

References

Glencross.B,D., Van Barnveld, R.J., Carter, C.G. and Clarke, S.M. (1999). On the path to a manufactured feed for farmed Southern Bluefin Tuna. World Aquaculture Society Journal 30: 42-46

Appendix E   Technique for making and administering an LHRHa slow-release cholesterol pellet

LHRHa slow release cholesterol pellets are used to stimulate ovulation in captive female dhufish and to maintain spermiation in captive males. The dose used for females is $50\mu g.kg^{-1}$ and for males is $10\mu g.kg^{-1}$. The pellets are made according to Lee *et al* (1986). Brief details are given below, however, Lee *et al* (1986) should be referred to for more details.

Making hormone pellets

Ingredients

- LHRHa – Sigma (L4513)

- Cholesterol – Sigma (C8667)

- Copha – available from supermarkets

- 50% ethanol (10 ml analytical grade ethanol plus 10ml distilled H_2O)

Equipment

- Pellet Mould – constructed of 10mm perspex with 1.6 mm holes drilled right through.

- Backing plate – a piece of perspex the same size as the pellet mould

- Ram – this is a metal rod that fits neatly inside the holes drilled in the pellet mould. This is used to compress the pellet mixture to form a pellet

- Small hammer – this is used to apply pressure to the ram to form the pellet

- Analytical balance to weigh individual pellets

- Drying oven set at $37^{o}C$

Procedure

1. Dissolve 4mg LHRHa in 0.5 ml of 50% ethanol in 50ml vial

2. Add 380mg cholesterol and mix well

3. Place mixture in the drying oven for 1h at 37'C

4. Add 20mg of cocoa butter (2 drops if molten) and mix thoroughly until its consistency is flaky.

5. Place a small amount of mixture into a hole in the pellet mould using a spatular.

6. Place the backing plate behind the mould and using the ram and hammer, compress the mixture. Keep adding the mixture and compressing until the mould is full and a pellet has been formed.

7. The pellet is then ejected from the mould by removing the backing plate and pushing the pellet out using the ram and hammer.

8. If the pellet falls apart add more copha to the mixture and try again. Take careful note of the weight of any extra copha additions, as this will be required for calculation of hormone concentration.

9. Calculate exact concentration of LHRHa in the pellet mixture and determine required weight of each pellet.

10. Cut each pellet to the size required (use analytical balance) and place individually in small labelled vials. Cut pellets to different sizes (within the range likely to be required). Vials should be labelled with date, amount of hormone/pellet and the weight of the pellet.

11. Pellets are stored in the freezer until use.

Administering pellets

Equipment
- scales
- 1.8mm internal bore needle (similar to a recycled PIT tag needle)
- PIT tag applicator
- foam restraint

Procedure

1. Sterilise in alcohol all equipment to be used in administering the pellet (needle, forceps, probe).

2. Anaesthetise fish

3. Weigh the fish and calculate the amount of hormone required

4. Select an appropriately sized pellet and load into a recycled PIT tag needle. Use forceps and/or a metal probe to do this,

5. Attach loaded needle to a PIT tag applicator or similar.

6. Place the anaesthetised fish ventral side up in a foam restraint.

7. Implant the pellet intraperitoneally by sliding the needle under a scale and through the flesh into the abdominal cavity, about 5 scales posterior to the pelvic fins (Fig A19). Once the needle is in place the plunger on the tag applicator is pushed to eject the pellet.
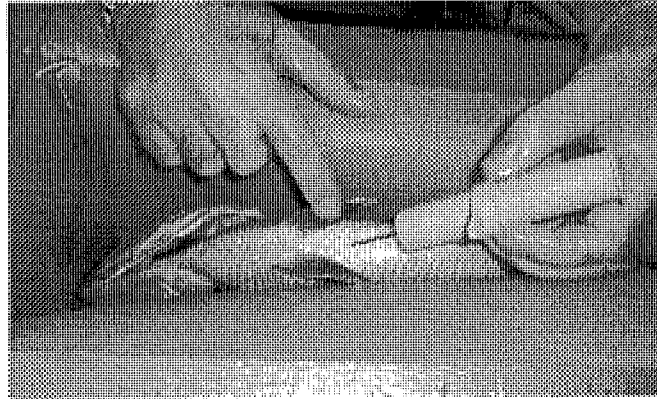
Figure A19: Implanting a slow-release cholesterol pellet into a sexually mature female held in a foam restraint

Reference

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

Appendix F   Stimulating in-tank spawning

Introduction

Procedures for hormonal induction of ovulation and subsequent stripping are sometimes the only means of obtaining eggs. Nevertheless, these procedures can negatively affect both the success of the procedure and the health of the broodstock. Stress imposed by capture and handling is known to affect reproduction in a wide range of species (reviewed in Pankhurst, 1998). Most techniques for administering hormones to induce ovulation and spawning require that the fish first be caught and handled. This is a major disadvantage as the stress may impair the efficacy of the hormone treatment. Furthermore, repeated stripping episodes can result in physical damage to the fish and in extreme cases, death. In addition, once eggs have ovulated, they must be stripped within the window of peak viability. Successful hand stripping in species with a short window of viability requires that the precise time of ovulation be predicted. If the fish spawns, however, the dependence on human interpretation is removed, as is the need to subject the fish to handling protocols. Therefore two pilot studies were conducted to investigate the use of hormones to stimulate in-tank spawning in dhufish and to eliminate handling during the process of hormonal treatment.

Part A

On two separate occasions during the 1996/97 spawning season, dhufish treated with a low dose of exogenous hormone released eggs in the tank which were subsequently fertilised by the resident male (FRDC report, June 1997). On one occasion the female was treated with a luteinising hormone releasing hormone analogue (LHRHa) pellet (5 $\mu g.kg^{-1}$) followed three days later by a LHRHa injection (5 $\mu g.kg^{-1}$). On the other occasion the female was treated with an injection of Ovaprim® (6 μg GnRH.kg^{-1}). In the following season we sought to confirm these results in a controlled manner using a low dose of LHRHa.

Part B

Dietary administration of hormones does not require pre-treatment handling and so avoids possible negative stress effects. This technique has not been widely investigated, however, it has been used successfully in several species including spotted seatrout (Cynoscion nebulosus; Thomas and Boyd, 1989), sable fish (Anoplopoma fimbria; Solar et al., 1990), goldfish (Carassius auratus; Suzuki et al, 1988), Thai carp (Puntius gonionotus; Sukumasavin et al., 1992) and coho salmon (Oncorhynchus kisutch; McLean et al., 1991). The dose required for successful oral administration is normally higher than is typically used in injections. Solar et al., (1990) reports that the dose required to induce ovulation in sable fish by oral intubation was 10-fold greater than for injection. Other workers report doses of between 0.5 and 3 mg.kg^{-1} (Thomas and Boyd, 1989). Although LHRHa administered by injection and slow-release

cholesterol based pellet has been demonstrated to be effective in inducing ovulation in dhufish (Pironet, and Neira, 1998, Appendix G and M this volume), the efficacy of administering LHRHa orally has not been investigated. Therefore a trial was conducted (within the natural dhufish spawning season) to test the efficacy of oral administration of LHRHa to stimulate ovulation and spawning in dhufish.

Methods

Part A

In January 1998, three females in a 50 m^3 tank were treated with 5 μg/kg LHRHa slow release cholesterol pellets (Appendix E), followed 3 days later by a 5 μg.kg^{-1} LHRHa saline-based injection. The remaining 2 females in the tank were treated identically but with a blank pellet and a saline injection (control). Similarly, in another tank (20m^3), two females were treated with LHRHa and two with a control. The dose used, however, was 10 μg.kg^{-1} (LHRHa or control equivalent) for both implant and injection. Following the injection, all fish were left undisturbed for a further 96 h post injection to enable time for release of the eggs. At 96h after the injection, the tanks were drained and the fish stripped to check for ovulation. Oocyte diameters prior to implant were between 550 and 700 μm. Tanks were fitted with egg nets on water overflows to trap any eggs released in the tank. Egg nets were checked daily for released eggs.

Part B

In one tank (50m^3), 2 females were fed their normal flesh diet with LHRHa injected into the flesh of the food so that each fish received a hormone dose of approximately 0.05 mg.kg^{-1}. In a separate tank (40m^3), two females were fed the same diet, however, the food was injected with a corresponding volume of saline solution (no LHRHa). The remaining three fish per tank were fed their normal diet without injection. This procedure was repeated weekly and then twice weekly to the same fish for 21 days. The dose of LHRHa (and corresponding saline dose) doubled each time (Table A11). The final dose used was 0.8 mg.kg^{-1} LHRHa on Day 21. Due to the requirement not to disturb the fish, no attempt was made to assess stage of maturation (by gonadal biopsy) of the females prior to the experiment.

All tanks were fitted with egg nets and these were checked daily for any eggs released into the tank. Observations were made daily (prior to feeding) of each fish to identify any change in the size of the abdomen which may indicate developing oocytes or ovulation.

Table A11:　　Schedule for oral administration of LHRHa to captive female dhufish.

Day of treatment	1	8	15	18	21
Dose (or equivalent) (mg LHRHa.kg^{-1})	0.05	0.1	0.2	0.4	0.8

Results/Discussion

Part A

In the 50m^3 tank (fish treated with LHRHa at 5 µg.kg^{-1} or control equivalent) there was no external evidence of ovulation (swelling of abdomen) or of spawning (eggs in egg collector). At 96h after treatment, ovarian biopsies showed that none of the LHRHa-treated fish ovulated, while one of the control fish ovulated. Less than 500 poor quality eggs were stripped from this fish. In the 20 m^3 tank, the abdomen of fish treated with LHRHa (10µg.kg^{-1}) swelled from 48h post injection, however no eggs were found in the collector, indicating that the fish did not spawn. At 96h post injection, the ovaries were excessively swollen and contained multiple batches of over-ripe ovulated eggs. Neither of the control fish ovulated.

The results from these trials indicate that the in-tank spawning that occurred in 1996/1997 are not readily reproducible. This is probably due the complex and interrelated nature of factors that affect the behavioural act of spawning (Pankhurst, 1998). These may include water temperature, tank dimension, tank-side disturbance, substrate availability and/or photoperiod. Clearly, it is not simply related to the correct hormone dose.

Part B

Eggs were not released in either the control or the LHRHa-treated tank, nor was there any visual evidence of ovulation (i.e. abdominal swelling) following any of the doses. The trial was terminated at this point since the high concentration of LHRHa required, high cost of the product and limited stocks of LHRHa precluded the use of higher doses.

The maximum dose tested in the current study was 0.8 mg.kg^{-1}. In other species the dose rate is slightly higher. Oral administration of LHRHa (1-2 mg LHRHa.kg^{-1}) of body weight) successfully induced spawning in spotted seatrout (*Cynoscion nebulosus*; Thomas and Boyd, 1989). Sablefish (*Anoplopoma fimbria*) were orally intubated with LHRHa at 1 mg/kg of body weight, followed by another dose at 0.5 mg.kg^{-1} 11 days later. This treatment resulted in three out of four treated sablefish ovulating 3 to 7 days after the second treatment (Solar *et al*, 1990). Therefore it is possible that the dose used in the current study was too low to induce a response. A higher dose should be tested.

Alternatively, these negative results may suggest that LHRHa is not readily absorbed through the gut, or that it is being broken down in the gut before absorption. Since the stage of maturation was not assessed prior to the experiment it is also possible that the gonads were not developed sufficiently for the LHRHa to have an effect. In order to confirm the cause of this lack of response, however, further work needs to be done. One way of approaching this problem would be to orally administer LHRHa in combination with a blood-sampling regime. Analysis of plasma 17β-estradiol and testosterone levels would indicate whether a gonadotropin surge was stimulated following oral administration of LHRHa, and hence whether LHRHa can be absorbed through the dhufish gut.

References

McLean, E.M., Parker, D.B., Warby, C.M., Sherwood, N.M. and Donaldsom, E.M. (1991). Gonadotropin release following oral delivery of luteinizing hormone-releasing hormone and its superactive analogue (des-Gly^{10}[D-Ala^6]LHRH ethylamide) to 17β-oestradiol-primed coho salmon, Oncorhynchus kisutch (Walbaum). Journal of Fish Biology 38: 851-858

Pankhurst, N.W. 1998. Reproduction. Pages 1-26 in. K. Black and A.D. Pickering editors. Biology of Farmed Fish. Sheffield Academic Press, Sheffield.

Pironet, F.N and Neira, F.J. 1998. Hormone induced spawning and development of artificially reared larvae of the West Australian dhufish, Glaucosoma hebraicum (Glaucosomatidae). Marine and Freshwater Research 49: 133-42

Solar, I.I., McLean, E., Baker, I.J., Sherwood, N.M. and Donaldson, E.M. (1990). Induced ovulation in sablefish Anoplopoma fimbria (Pallas, 1811), following oral administration of des Gly^{10}(D-Ala^6) LHRH ethylamide. Fish Physiology and Biochemistry 8: 497-499

Suzuki, Y., Kobayashi, M., Nakamura, O., Aida, Katsumi and Hanyu, I. (1988). Induced ovulation of the goldifsh by oral administation of salmon pituitary extract. Aquaculture 74: 379-384

Sukumasavin, N., Leelapatra, E., McLean, E. and Donaldson, E.M. (1992). Orally induced spawning of Thai carp (Puntius gonionotus Bleeker) following co-administration of des Gly10(D-Arg6) sGNRH ethylamide and domperidone. Journal of Fish Biology 40: 477-479

Thomas, P. and Boyd, N.W. (1989). Dietary administration of an LHRH analogue induces spawning of spotted seatrout (Cynoscion nebulosus). Aquaculture 80: 363-370

Appendix G   The response of acclimated female dhufish to implant with LHRHa slow-release pellet

Introduction

When fish are caught or held in captivity, natural ovulation may not occur. It is therefore often necessary to induce ovulation with exogenous hormone treatments. LHRHa has been shown to stimulate oocyte maturation and ovulation in gilthead seabream (Zohar *et al*, 1988), red seabream/snapper (*Pagrus auratus*; Battaglene, 1995) and salmonids (reviewed in Donaldson and Devlin, 1996). Although recent trials have suggested that LHRHa, may induce ovulation in dhufish (Pironet and Neira, 1998, Appendix M), this remains to be proved in a controlled manner. Therefore an experiment was conducted to determine the effect of a slow-release cholesterol pellet containing LHRHa on ovulation in dhufish.

Methods

During January and February of 1999 and 2000, tanks containing acclimated dhufish were drained to knee depth and fish anaesthetised with 2-phenoxyethanol (0.3ppt). An ovarian biopsy was conducted on each female to determine stage of sexual maturity. Sexually mature females (ovaries containing vitellogenic, hydrating or ovulated oocytes; oocyte diameter >400µm) were weighed and then implanted intraperitoneally with a slow-release cholesterol-based pellet containing either 0 (control; n=3) or 50 µg.kg^{-1} LHRHa (n=10). The pellet implants were made according to Lee *et al* (1986), and contained 95% cholesterol and 5% copha oil (Appendix E). Implants were administered using a large bore needle with an internal shaft. LHRHa-treated fish weighed 6.67 ± 0.27kg, while control fish weighed 5.53 ± 0.98kg.

After treatment the fish were then placed in a 4000L tank and regular ovarian biopsies were conducted (following light anaesthesia) to assess the stage of oocyte development and to predict the time of ovulation. These were conducted at least every 24h. As soon as ovulation was detected, the fish was anaesthetised deeply for stripping. Once anaesthetised, the abdominal region of the fish was rinsed with freshwater and gently towel dried to remove traces of salt water and anaesthetic. The fish was then stripped by applying pressure along the abdomen, beginning at the front of the fish (behind the pectoral fins) and moving toward the vent. Sperm and seawater was added to the eggs, followed by gentle mixing. Sperm supply in dhufish is limiting and sperm quality is highly variable. Therefore sperm was obtained from whatever source was available including sperm stripped from freshly caught captive males, or fish caught from the wild. Water temperature was not controlled and ranged between 21.0 and 24.5°C.

From ovulation (T=0h post-ovulation fish were stripped every 24h. The trial was concluded at 96 h post capture. At each stripping, the number, viability and fertility of eggs were determined

volumetrically. Eggs were stocked into 43L incubation cones for hatching. Once hatched the number of larvae were counted volumetrically.

Paired T-tests were used to compare the first and 2nd successive ovulation from each ovulating fish, in terms of numbers of eggs produced, viability and fertilisation. Other statistical tests were not conducted due the irregularity of the data.

Results/Discussion

Oocyte diameters at the time of treatment for all fish were between 500 and 610μm. In the control treatment oocyte diameters remained similar throughout the experiment (Fig A20). In the LHRHa treatment, mean oocyte diameter was 800μm by 48h after treatment and remained at greater than 800μm through to the end of the experiment.

Sixty percent of LHRHa treated fish ovulated during the 96h of the experiment, while none of the control fish ovulated. Few eggs were released on the first ovulation for each fish (<300 eggs). These small ovulations were ignored and only ovulations of more than 1000 eggs were included in the following data. Twenty percent of LHRHa-treated fish first ovulated between 24 and 48h post treatment. Thirty percent of LHRHa-treated fish first ovulated between 48 and 72h post treatment. Twenty percent first ovulated between 72 and 96h post treatment. Forty percent of fish failed to ovulate within the 96 h of the experiment. One of these died at 48h post treatment but had hydrating oocytes at death. Another fish had hydrated oocytes at 96h and ovulated 7h later (beyond the time frame of this experiment).
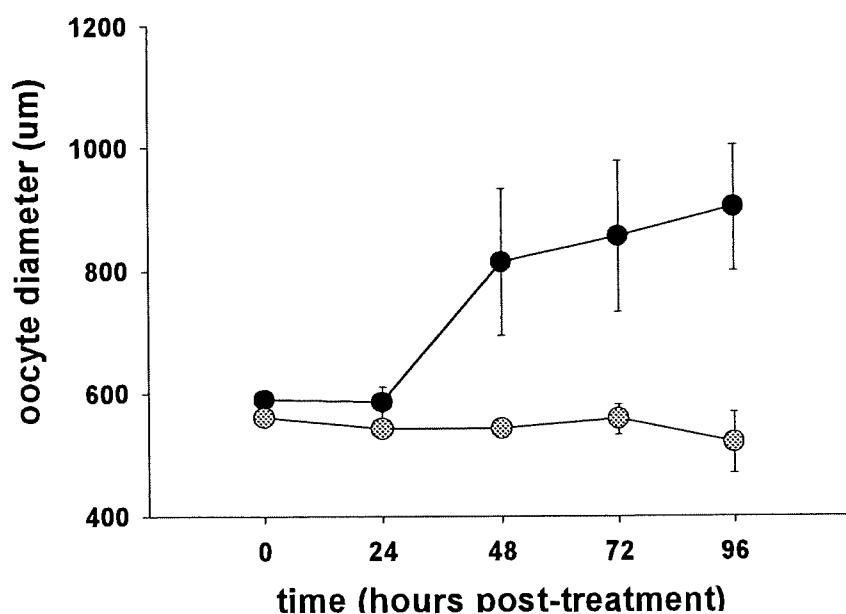
Figure 20: Change in mean oocyte diameter with time in acclimated female dhufish following treatment with an LHRHa slow release cholesterol pellet (50μg.kg^{-1}; black circle; n=10) or a control pellet (0 μg.kg^{-1}; grey circle; n=3). Values are means ± SE

Of the fish that ovulated, all fish ovulated at least twice; 42% ovulated twice and 58% ovulated three times. As the time of first ovulation varied among fish, for the purposes of further analysis, first ovulations from each fish were grouped together, as were second or third successive ovulations. At each successive ovulation an average of between 80,000 eggs and 120,000 eggs were stripped (Fig A21). The largest single ovulation was approximately 300,000 eggs, while the smallest was 17,000. The most number of eggs stripped from any one female during the experiment was 571,000 (in 3 ovulations), while the fewest number was 64,809 (in 2 ovulations). Viability of eggs in first ovulations was 95%. This decreased to 68% by the second ovulation (P<0.01). By the third ovulation viability was only 37%. Fertilisation success of viable eggs was low, with an average of less than 40% of the first, second and third ovulations being fertilised. Maximum fertilisation was 95%, while the lowest was 0%. Hatch was 44% in eggs from first ovulations. By the second and third ovulation, hatch was 24 and 26% respectively, almost half that of the first ovulation, however large variation prevented detection of a significant difference.
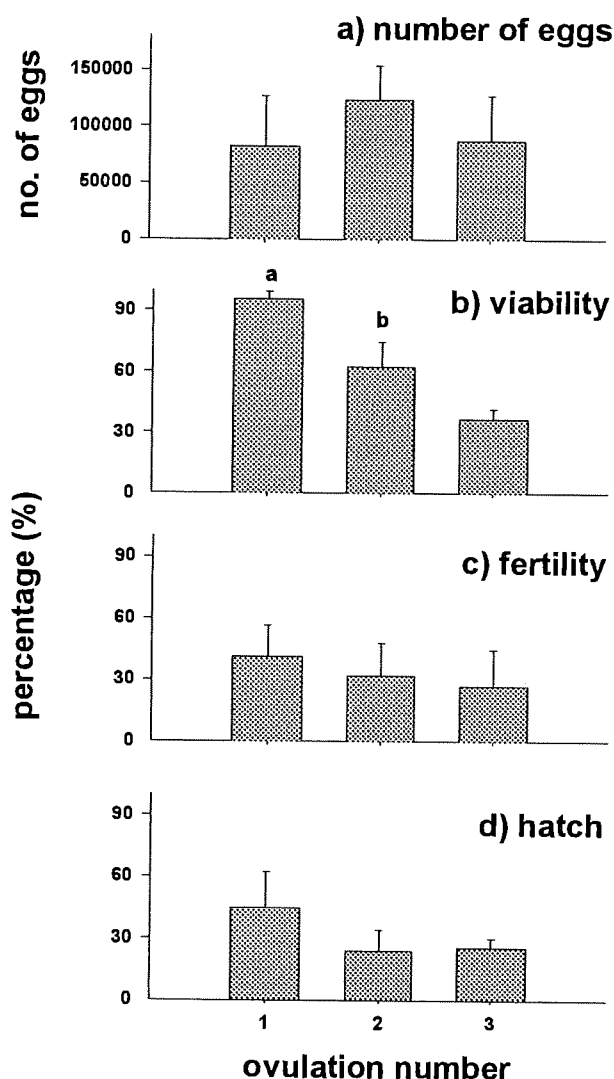
Figure A21: The number, % viability, % fertilisation, % hatch of eggs stripped from acclimated female dhufish following treatment with an LHRHa slow release cholesterol pellet. Different letters indicate a significant difference between first and second ovulations

Therefore LHRHa at a dose of 50μg.kg^{-1} stimulated oocyte development in dhufish, with 60% of fish responding to the treatment, while none of the control fish did. If the fish that died and the one that ovulated a few hours after the conclusion of the experiment are considered, 80% of the treated fish ovulated. This makes the LHRHa pellet at 50μg.kg^{-1} highly effective at stimulating ovulation in acclimated female dhufish. The time to first ovulation varied widely from 24 through to 96 h post treatment, making prediction of the time of injection impossible, without regular monitoring. The water temperature, however, was not controlled and varied between 21.0 and 24.5°C. This is likely to have influenced the latency period from treatment to ovulation. This should be addressed in further trials

All of the fish which ovulated, did so two or three times within the 96 h from treatment. Although the number of eggs produced at each successive ovulation did not appear to vary, the viability of the eggs decreased from the first ovulation to the second ovulation and presumably from the second to the third (although this was not tested statistically). This reduction in viability could be due to errors made in detecting the time of ovulation, or due to a reduction in egg quality with increasing time from treatment. Neither fertilisation (as a percentage of viable eggs) or hatch (as a percentage of fertilised eggs) decreased with successive ovulations. Fertilisation and hatch were low at each ovulation. Although fertilisation is also attributable to egg quality, sperm supply and quality in dhufish is limiting and an inadequate supply of sperm was often the primary cause of low fertilisation. When good quality sperm was available in sufficient quality and this coincided with good quality eggs, fertilisation could be as high as 95%. This emphasises the importance of continuing research in to securing a supply of good quality sperm, including refining techniques for cryopreserving sperm (Appendix L).

References

Battaglene, S.C.(1995). Induced ovulation and larval rearing of four species of Australian marine fish. *PhD. Thesis,* University of Tasmania. 215p

Donaldson, E.M. and Devlin, R.H. (1996). Uses of biotechnology to enhance production. In *Developments in Aquaculture and Fisheries Science. Principles of salmonid culture* (eds W.Pennel and B.A. Barton). Elsevier, Amsterdam pp 969-1021

Pironet, F.N and Neira, F.J. 1998. Hormone induced spawning and development of artificially reared larvae of the West Australian dhufish, *Glaucosoma hebraicum* (Glaucosomatidae). Marine and Freshwater Research 49: 133-42

Zohar, Y., Pagelson, G. and Tosky, M. (1988). Daily changes in reproductive hormone levels in the female gilthead seabream *Sparus aurata at the spawning period. In* Reproduction in Fish – Basic and Applied Aspects in endocrinology and genetics. pp 119-125. IRNA Press, Paris.

Appendix H   Stimulating ovulation from dhufish caught from the wild during the spawning season

Introduction

At the ADU, fertilised eggs are normally obtained by artificially stimulating acclimated dhufish (ie caught from the wild and held in captivity for several months prior to the spawning season; Appendix G). The number and quality of the eggs obtained however is highly variable. There are many factors that may determine egg quality including nutrition and husbandry conditions (Bromage, et al, 1994; Pankhurst, 1998). In stress sensitive species, procedures such as routine tank maintenance, feeding or any other tank-side activity may be sufficient to induce increases in plasma cortisol levels and decreases in plasma gonadal steroid levels (Cleary et al, 2000a). Reduced plasma gonadal steroid levels have been shown to impair the response to hormone therapy in some species (Haddy and Pankhurst, 2000; Cleary et al, 2000b). Furthermore, broodstock nutrition also affects egg quality. As dhufish culture is in its infancy, little is known about broodstock nutrition. The broodstock are fed a diet of high quality fish flesh (including squid, whiting, garfish, prawns, and mullet) supplemented with vitamins and minerals (Appendix C & D). Every effort has been made to optimise this diet, however, in the absence of the facilities and resources to conduct trials, it is possible that this diet is not as good as that consumed in the wild. Therefore it is likely that the problem of poor egg quality in dhufish is a direct consequence of the culture environment.

Fish in the wild feed on a natural diet and are less likely to be exposed to chronic or acute stress during the critical period of vitellogenesis than captive fish. Therefore the use of sexually mature fish caught from the wild during the spawning season and injected with hormones at the time of capture may improve egg quality. In addition, the use of fish caught from the wild during the spawning season precludes the need to hold captive stock year round. Therefore in this pilot study, the potential of using dhufish caught from the wild during the spawning season was assessed. A variety of hormone treatments were tested: luteinising hormone releasing hormone analogue (LHRHa; injection or slow-release cholesterol pellet) or human chorionic gonadotropin (hCG; injection only). Both hCG and LHRHa are commonly used to induce ovulation in a variety of marine finfish and have also been used successfully to induce ovulation in captive dhufish (Pironet and Neira, 1998, Appendix G this volume).

Methods

During the natural spawning season (December to April) between December 1998 and April 2000, dhufish were caught by handline or dropline from waters off the Perth metropolitan region. Fish were caught from depths ranging from 10 to 60m. Fish larger than 10kg were rejected as they are too difficult to handle. The swimbladder of suitably sized females was vented and the fish placed ventral side up in a foam restraint. An ovarian biopsy was conducted to determine

the stage of sexual maturity. Sexually mature females (ovaries containing vitellogenic, hydrating or ovulated oocytes; oocyte diameter > 400μm) were retained. Total length was measured and body weight estimated based on length to weight ratio described for dhufish caught from the wild in earlier years of this project. For convenience this data was tabulated to enable rapid conversion of weights into dose rates (eg Table A12). The fish were injected with either 200 IU hCG.kg^{-1}, 1000 IU hCG.kg^{-1}, >1000 IU hCG.kg^{-1}, 100 μg LHRHa.kg^{-1} (saline-based injection), 10ug LHRHa.kg^{-1} (slow-release cholesterol pellet or a saline injection/blank pellet (control). LHRHa injections and pellets were pre-prepared and stored in appropriately sized aliquots in the freezer. These were kept frozen on dry ice while at sea, and thawed immediately prior to use. HCG was in the form of Chorulon®. The undiluted hCG powder was stored on ice and diluted according to manufacturers instructions immediately prior to use (see section 5.2).

Table A12 :   Length-weight-dose conversion used at sea to determine correct dose for injectiom. The dose rate for hCG (10000IU.kg^{-1}) using a stock solution of 4000IU.ml^{-1} is given as an example.

Min FL (cm)	Max FL (cm)	Est Weight (kg)	hCG (4000IU.ml^{-1}) (ml)
	35.2	0.5	0.13
35.3	40.8	1	0.25
40.9	45.4	1.5	0.38
45.5	49.3	2	0.50
49.4	52.9	2.5	0.63
53.0	56.1	3	0.75
56.2	59.0	3.5	0.88
59.1	61.8	4	1.00
61.9	64.4	4.5	1.13
64.5	66.9	5	1.25
67.0	69.3	5.5	1.38
69.4	71.6	6	1.50
71.7	73.7	6.5	1.63
73.8	75.8	7	1.75
75.9	77.9	7.5	1.88
78.0	79.8	8	2.00
79.9	81.7	8.5	2.13
81.8	83.6	9	2.25
83.7	85.4	9.5	2.38
85.5	87.1	10	2.50
87.2	88.9	10.5	2.63
89.0	90.5	11	2.75
90.6	92.2	11.5	2.88
92.3		12	3.00

After treatment, fish were placed in a covered 200-250L tank supplied with oxygen and regular water exchange for transport to the ADU. At the ADU the fish were transferred to covered 4000L tanks, supplied with flow-through water and aeration. Every 24 h from capture, fish were anaesthetised lightly and an ovarian biopsy performed. If the developing oocytes were 800μm or greater, biopsies were performed more regularly to enable prediction of the time of ovulation and hence successful stripping.

Once ovulation was detected, ovulating females were anaesthetised and stripped. Sperm, collected immediately prior from live acclimated males or from wild-caught males killed and stored on ice, was used to fertilise the eggs. Egg quality was assessed by volumetrically estimating percentage viability, fertilisation and hatch.

Results/Discussion

Capture of sexually mature females

Seventy-two male and sixty-five female dhufish were caught. Of the females, forty-eight were immature, and nineteen were sexually mature (eleven vitellogenic and twelve hydrating or ovulated). Three of these died shortly after capture. The remainder were treated with hormones and transported back to FMC.

Following hormone treatment it is necessary for the fish to survive for at least 96 h to enable the female to respond to the hormone and ovulate. Survival of sexually mature females to 96h after capture was 72% in fish caught in water 30m or shallower and only 17% in fish caught in waters deeper than 30m. Therefore, for greatest success, sexually mature females must be caught in shallow water.

A total of forty-six fishing trips were made during the natural spawning season of the dhufish (December to April) during 1998, 1999 and 2000. The twenty-three sexually mature females caught in water depths across the full range of water depths fished in (between 10 and 60m), with twelve being caught in shallow water (<30m) and sixteen in deep water (>30m). Averaged across all fishing days, the number of mature fish caught per fishing trip in deep water, was more than twice as high from deep water than shallow water. An average of 0.88 mature fish were caught per fishing trip in deep water compared with 0.41 in shallow water. Therefore, although sexually mature fish were caught in waters above and below 30m in depth during the spawning season, it took approximately twice the fishing effort to catch fish in shallow water than the deeper water. With increased local knowledge and further understanding of the behaviour of spawning fish, the fishing effort may be reduced.

Averaged across all three years sampled, the number of mature females caught per fishing trip in shallow water was highest during December with 0.80 mature females caught per fishing trip (Table A13). From January through to April less than 0.40 mature females were caught per

fishing trip in shallow water. This data suggests that fishing for sexually mature females in shallow water should commence early in the spawning season. During late spring and summer the location of the dhufish population is associated with the annual migration of the crayfish "whites" (soft post-moult crayfish; Ron Lopresti, Revilo PTY LTD, 2000, pers com, 2000). In the Fremantle area, the crayfish normally (but not always) first appear on approximately the 7-10th December and are abundant in near shore areas until mid-January. From mid-January the "whites" begin to migrate to deeper waters. As crayfish is a common food for dhufish, it is widely believed that dhufish populations follow the "whites" to deeper water. This is consistent with data in the present study showing that sexually mature females were easier to catch in shallow water in December than in other months. It should be noted that "whites" run later in the Fremantle than they do waters to the north. Therefore fishing for sexually mature females in shallow waters to the north of Perth should begin earlier in the season.

Table A13:   Capture rate of sexually mature dhufish caught from the wild in shallow waters (<30m)

	# trips	# mature females/trip
December	5	0.80
January	8	0.38
February	10	0.30
March	4	0.25
April	3	0.33
Combined	30	0.41

Hormone induction of ovulation

Three sexually mature females were treated with a blank pellet or injection (control). Two of these had hydrating oocytes (ie > 800μm) at capture and ovulated while being transported to the ADU (Table A14). Both of these fish were stripped at the ADU, and although one produced 18,500 eggs, no eggs were viable. The poor viability is probably due to the eggs being stripped outside of the window of peak viability, as the fish had ovulated at sea (Appendix I). These fish did not ovulate again. The oocytes of Fish # 1 decreased from 1200 μm to 600 μm by 48h post-treatment (Fig A22a). Fish # 2 died at 48h post-treatment when its oocytes were still approximately 100μm. The third control fish had oocytes of 500 μm and these did not develop throughout the experiment. This data suggests that even if ovulating females are caught from the wild, hormone treatments will be required to stimulate ovulation on successive days. In

addition, in order to utilise eggs ovulating shortly after capture, it will be necessary to be equipped to strip ovulating females at sea.

Two fish were injected with LHRHa ($100\mu g.kg^{-1}$). One of these had hydrating oocytes at capture, however, failed to ovulate before dying 6 h after capture (Table A14). The other fish failed to respond to the treatment and oocyte diameters remained less than $600\mu m$ (Fig A22b). Three fish were treated with an LHRHa slow-release cholesterol pellet ($10\mu g.kg^{-1}$). One fish, having vitellogenic oocytes ($592\mu m$) failed to respond to the treatment and oocyte diameters remained similar throughout (Table A14; Fig A22c). Two fish had hydrated oocytes at capture. One of these died within 6 h of capture and failed to ovulate in this time. The other did not ovulate and oocyte diameters gradually decreased to $700\mu m$ by 96h post-capture. Therefore

Table A14:   The initial oocyte diameter, number of ovulations , the number of ovulated eggs and the number of viable eggs of individual female dhufish caught from the wild and treated with a control treatement, LHRHa injection ($100\mu g.kg^{-1}$), LHRHa pellet ($10\mu g.kg^{-1}$), hCG injection ($200IU.kg^{-1}$), hCG injection ($1000IU.kg^{-1}$) or hCG injection ($>1500IU. kg^{-1}$).

Treatment	Fish ID	Initial oocyte diameter	No. ovulations	Total no. ovulated eggs	No. viable eggs
Control	1	1124	1	18500	0
	2	1010	1	300	0
	3	500	0	0	0
LHRHa injection ($100\mu g.kg^{-1}$)	4	506	0	0	0
	5	1050	0	0	0
LHRHa pellet ($10\mu g.kg^{-1}$)	6	1052	1	33800	14196
	7	592	0	0	0
	8	1104	2	18000	0
HCG ($200IU.kg^{-1}$)	9	708	0	0	0
	10	560	0	0	0
hCG ($1000IU.kg^{-1}$)	11	659	2	200	0
	12	588	4	63250	46860
	13	1000	4	105550	44737
	14	1100	4	71365	48276
hCG ($>1500IU. kg^{-1}$)	15	1036	4	113000	84733
	16	1200	3	17356	6729

LHRHa at the rates tested (by injection or pellet) was not a successful treatment. Given the subsequent success of LHRHa pellet at 50 µg.kg^{-1} in acclimated females (Appendix G), it is likely that the doses tested were too low, and so higher doses should be tested.

Two fish were treated with hCG at 200IU.kg. Both of these had vitellogenic oocytes (550-710 um) at capture and failed to ovulate during the next 96h. Four fish were treated with hCG at 1000IU.kg. Two of these had hydrated oocytes (1000 and 1100µm) at capture, while two had vitellogenic oocytes (588 and 659um). The former 2 fish ovulated, producing a total of 63,173 (59435 viable) and 71365 (48,276 viable) eggs over four successive strippings. The remaining 2 fish first ovulated on days 2 and 3 after capture, one fish producing 92,850 (51945 viable) eggs over four successive strippings, while the other fish produced only 200 (0 viable) eggs. Two fish were treated with hCG at >1500 IU/kg. Both of these had hydrated oocytes at capture, and
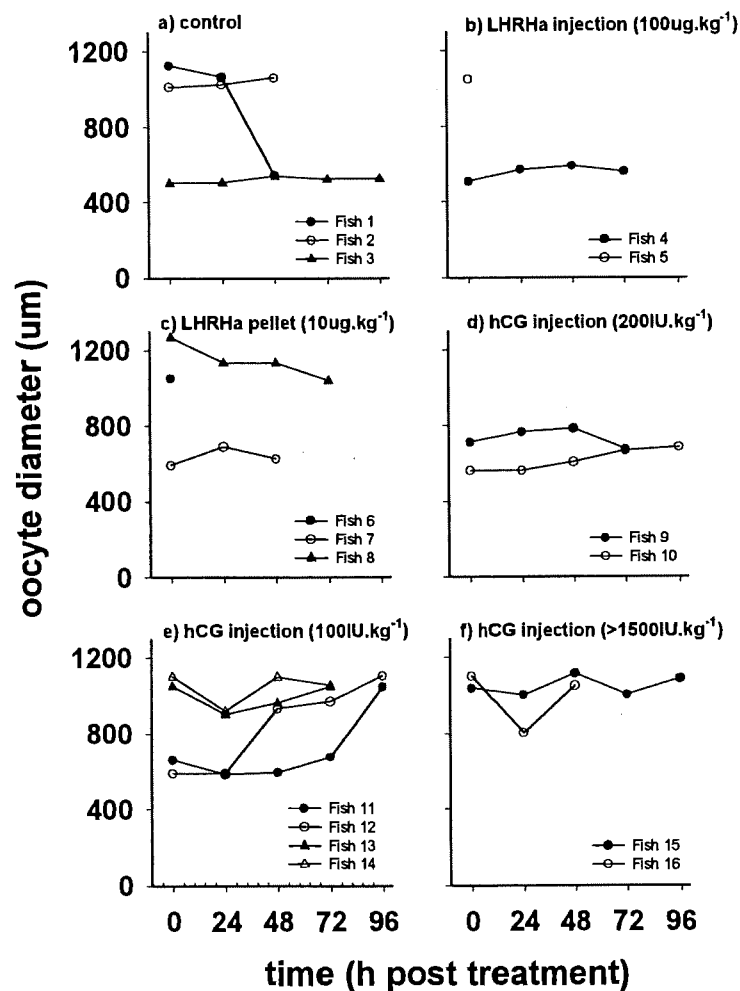
Figure A22 : Change in oocyte diameter with time from treatment of female dhufish caught from the wild and treated at capture with a) control treatement, b) LHRHa injection (100µg.kg^{-1}), c) LHRHa pellet (10µg.kg^{-1}), d) hCG injection (200IU.kg^{-1}), e) hCG injection (1000IU.kg^{-1}) or f) hCG injection (>1500IU. kg^{-1}).

ovulated on successive days. One fish produced 142,400 (95,420 viable) eggs, while the other produced 17,356 (6729 viable) eggs. Twenty-four hours after treatment, detection of ovulation became difficult. There ceased to be a distinct class oocytes proceeding to ovulation. Instead some oocytes were beginning hydration, while others were completing it. This is reflected in the poor viability (<50%) as no matter when stripping was conducted, both old and new oocytes were stripped. Gwo *et al* (1993) reported a similar problem, stating that hCG caused generalised hydration of all oocytes in the ovary, while LHRHa caused selective hydration of oocytes at a specific stage of development. This problem, however, was not observed following treatment with hCG at the lower dose of (1000IU.kg^{-1}).
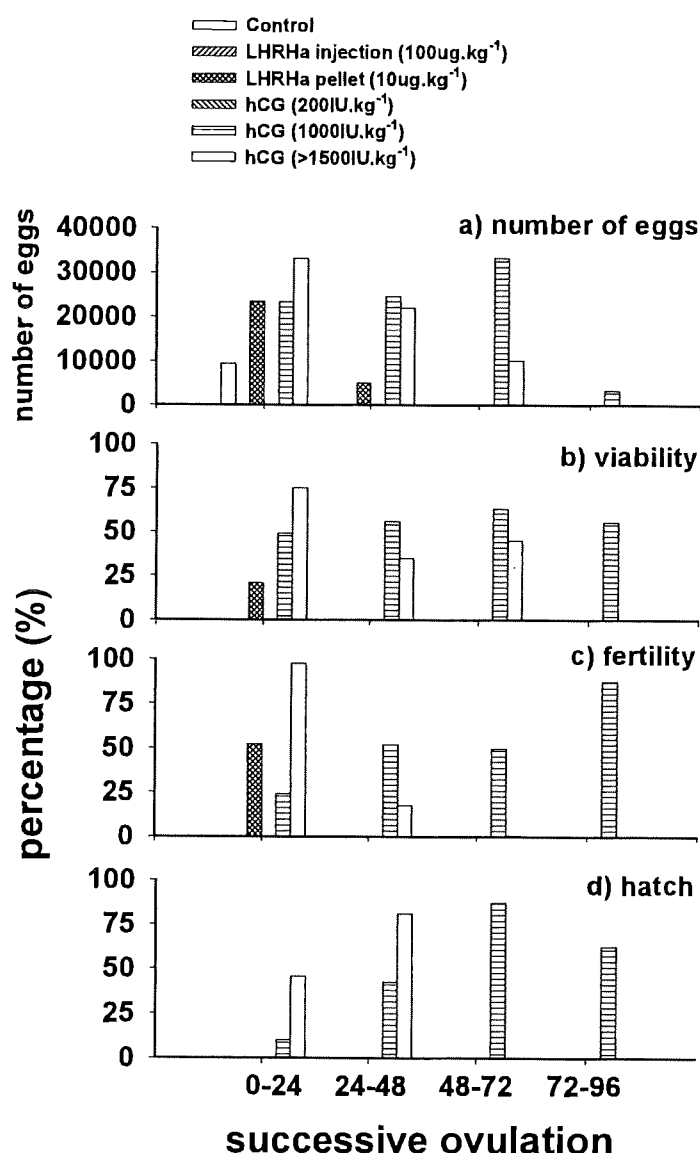
Figure 23: Mean number, % viability, % fertilisation and % hatch of eggs stripped from female dhufish caught from the wild treated with a control treatement, LHRHa injection (100µg.kg^{-1}), LHRHa pellet (10µg.kg^{-1}), hCG injection (200IU.kg^{-1}), hCG injection (1000IU.kg^{-1}) or hCG injection (>1500IU. kg^{-1}).

Although ovulation was detected in 5 out of 6 of the treatments tested, in the majority of the treatments the fish only ovulated once and this was within 24h of capture. HCG at both 1000IU.kg^{-1} and 1500IU.kg^{-1}, however, stimulated multiple successive ovulations (data not shown).

The maximum mean number of eggs from any 24h period for any treatment was approximately 30,000 (1000IU.kg^{-1}hCG at 48-72h post-treatment and >1500 IU.kg^{-1}hCG at 0-24h post treatment; Fig A23a). The largest single ovulation was 42,400 eggs (1000IU.kg^{-1}hCG; 16 h post treatment). The most eggs stripped from any one female was 113,000 over 4 successive strippings (>1500IU.kg^{-1}hCG). Mean viability ranged from 0 to 75% (Fig A23b). Mean fertility (as a percentage of viable eggs) ranged from 20 to 95% *Fig A23c). Mean hatch (as a percentage of fertilised eggs) was highly variable with mean hatch being between 0 and 90% (Fig A23d). Therefore the number and quality of the eggs produced was highly variable. HCG at 1000IU.kg^{-1}, however, gave consistent results with the mean number of eggs being 20,000 to 30,000 and viability being approximately 50%.

Therefore fertilised eggs can be obtained from females caught from the wild during the spawning season and treated at capture with hCG at 1000IU.kg^{-1}. The success of obtaining good quality eggs which subsequently were fertilised and hatched from dhufish caught from the wild during the spawning season is an important development in securing a reliable supply of good quality eggs. In addition to the potential negative effects of the culture environment on egg production in captive fish, maintaining broodstock year round for a short spawning season once a year is expensive (requires large tanks, labour and high quality feed) and risky. Further refinement to the techniques for obtaining eggs from fish caught during the spawning season may open the possibility to not hold captive broodstock, but rather fish each summer for broodstock. A cost-benefit analysis would need to be done to justify this. This technique also provides the possibility for establishing new ventures or recovery after disease.

References

Bromage, N., Bruce, M., Basavaraja, N., Rana, K., Heilds, R., Young, C., Dye, J., Smith, P., Gillespie, M., and Gamble, J. (1994). Egg quality determinants in finfish: the role of overripening with special reference to the timing of stripping in the Atlantic halibut *Hippoglussus hippoglossus*. Journal of the World Aquaculture Society 25: 13-21

Cleary, J.J., Pankhurst, N.W. and Battaglene, S.C. (2000a). The effect of capture and handling stress on plasma steroid levels and gonadal condition in wild and farmed snapper, *Pagrus auratus* (Sparidae). Journal of the World Aquaculture Society in press

Cleary, J.J., Battaglene, S.C. and Pankhurst, N.W. (2000b). Capture and handling stress affects the endocrine and ovulatory response to exogenous hormone treatment in snapper, *Pagrus auratus* in review

Gwo, J.C., Strawn, K., Arnold, C.R. (1993). Induced ovulation in Atlantic croaker (sciaenidae) using hCG and an LHRH analog: a preliminary study. Theriogenology 3: 353-361

Haddy, J.A. and Pankhurst, N.W. (2000) The efficacy of exogenous hormones in stimulating changes in plasma steroids and ovulation in wild black bream *Acanthopagrus butcheri* is improved by treatment at capture. Aquaculture in press

Pankhurst, N.W. 1998. Reproduction. Pages 1-26 *in*. K. Black and A.D. Pickering editors. Biology of Farmed Fish. Sheffield Academic Press, Sheffield.

Pironet, F.N and Neira, F.J. 1998. Hormone induced spawning and development of artificially reared larvae of the West Australian dhufish, *Glaucosoma hebraicum* (Glaucosomatidae). Marine and Freshwater Research 49: 133-42

Appendix I   Determining the window of post-ovulatory viability

Introduction

All externally fertilising species have a window of post-ovulatory viability after which there is a time dependant decline in egg viability (reviewed in Bromage *et al*, 1994). The period of peak fertility varies among species, from several hours (eg snapper *Pagrus auratus*; Hobby and Pankhurst, 1997) to several days to weeks (eg rainbow trout; Bromage *et al*, 1994). Reliable stripping of good quality eggs is dependent on a correct estimate of the length of this window, for the species in question. The length of the window of postovulatory viability for dhufish is unknown. The capacity to accurately estimate this window is beyond the scope of this project, as it would require large numbers of fish. Nevertheless, an experiment was conducted to gain an indication of the length of the window viability of dhufish oocytes.

Methods

Six captive dhufish were treated with a slow release cholesterol pellet containing LHRHa at $50\mu g.kg^{-1}$ (Appendix E). Each fish was then placed in a 4000L tank and checked regularly for ovulation by ovarian biopsy. As soon as ovulation was detected (eggs flowing freely; T=0 h post-ovulation), a portion of the eggs were stripped. Eggs were collected and mixed with sperm stripped from a captive male (if available). Sperm was stripped prior to stripping the female at 0h and stored in a syringe on ice for later strippings. Seawater was added and the eggs were gently mixed to disperse the spern. Egg quality was determined by taking a sample of eggs in a petri dish filled with seawater and examining them under a dissecting microscope to determine the percentage of eggs which were round, clear and buoyant. Two hours after adding the sperm, fertility was assessed by recording the percentage of eggs undergoing cell division. From T=0 h post ovulation, the fish were anaesthetised, and stripped as above every 3 h for 9h, or until fertilisation was less than 5%. Water temperature ranged between 21.5 and 23°C.

Results/Discussion

The mean percentage of round, clear, buoyant eggs was 75% at the time of ovulation (0h; Fig A24). By 3h after ovulation, this was less than half, with 40% of eggs being round clear and buoyant. The mean percentage of round clear and buoyant eggs further decreased to be less than 10% by 9h post ovulation. Due to an unreliable supply of sperm from male dhufish, sperm was only available to fertilise eggs from three females. Mean fertilisation success was 70% at ovulation. By 3h post-ovulation, 55% of eggs were fertilised and by 6h, only 35% of eggs were fertilised. By 9h post-ovulation fertilisation was virtually 0%.

The window viability in dhufish appears to be short. In a similar study on snapper, *Pagrus auratus*, fertilisation fell below 50% between 6 and 9 h post ovulation (Fig A24; Hobby and

Pankhurst, 1997). In the current study, the percentage of round, clear and buoyant eggs was less than 50% before 3h post ovulation, and fertilisation was less than 50% shortly after 3h. In the present study sperm was stripped prior to stripping the female for the first time, and stored in a syringe for use at later strippings. This was done as dhufish produce very small amounts of sperm (<1ml at any one time) and this was the only way of ensuring sperm was available for the latter strippings. This method, however, opens up the possibility that the decline in fertilisation may be at least partly affect by a decline in sperm quality in the 9 h from stripping. The fact that the percentage of round clear and buoyant eggs decreased concomitant with fertilisation, however, suggests that this is not the case. The short window of viability for dhufish means that care must be taken in induced ovulation and stripping protocols to accurately detect the time of ovulation and to strip as soon as possible after.
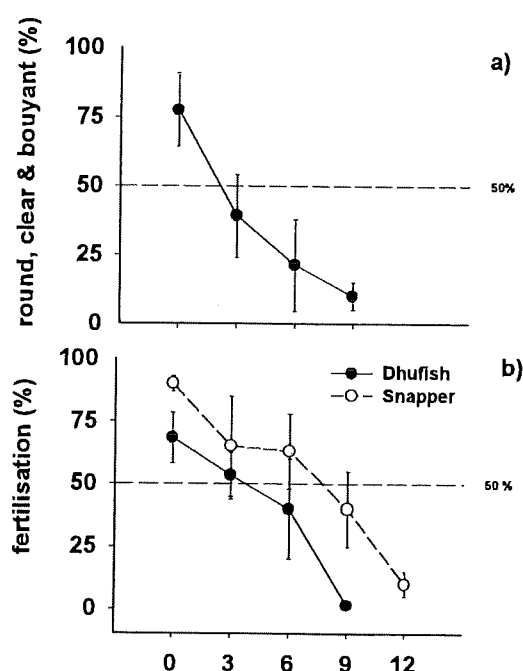
Figure A24: The percentage of a) round, clear and buoyant eggs and b) percentage fertilisation of eggs stripped from ovulated dhufish at 0, 3, 6, 9 and 12 h after ovulation (n=3-6; black circles). The percentage fertilisation of eggs stripped from snapper, *Pagrus auratus*, at 0, 3, 6, 9, and 12h after ovulation is presented for comparison (clear circles; from Hobby and Pankhurst, 1997). Values are means ± SE

References

Bromage, N., Bruce, M., Basavaraja, N., Rana, K., Heilds, R., Young, C., Dye, J., Smith, P., Gillespie, M., and Gamble, J. (1994). Egg quality determinants in finfish: the role of overripening with special reference to the timing of stripping in the Atlantic halibut *Hippoglussus hippoglossus*. Journal of the World Aquaculture Society 25: 13-21

Hobby, A. and Pankhurst, N.W. (1997). Post-ovulatory egg viability in the snapper *Pagrus auratus* (Sparidae). Marine and Freshwater Research 48:385-9

Appendix J   The effect of LHRHa implant on acclimated males

Introduction

Although male dhufish can spermiate in captivity if given sufficient acclimation time, sperm supply is currently limiting the success of the artificial propagation program for dhufish. Only the dominant males will spermiate in any particular tank and the volume of sperm produced is normally less than 1ml. Although there is some indication that the most beneficial treatment is a low dose slow-release LHRHa pellet, this information is somewhat anecdotal and it remains to be tested in a controlled manner. Therefore this experiment was conducted to examine the effect of an LHRHa slow-release pellet on the volume of sperm produced and sperm viability

Methods

Twenty male dhufish were caught from the wild and acclimated in a number of 20,000-L tanks at the ADU; each tank containing from 2 to 6 males and up to 5 females. During three successive spawning seasons (December – April; 1997/1998, 1998/1999 and 1999/2000) tanks were drained to knee depth and the fish herded into a crowd where they were anaesthetised with 2-phenoxyethanol (3ppm) according to standard practice at the ADU. The abdomen of the fish was rinsed in fresh-water to remove salt-water and anaesthetic residues. The fish were then stripped by applying pressure along the abdomen toward the vent. All sperm released was collected in a sterile hypodermic syringe and stored on ice. Each fish was then weighed, measured and implanted with a slow-release cholesterol pellet containing either 0 or 10 µg LHRHa.kg^{-1} (Appendix E). The fish were then released into 4000L tanks (2-3 fish per tank; 1 treatment per tank) where they remained undisturbed until Day 3 post-implant. On day 3 and 6 post-implant, each tank was drained and the fish anaesthetised, and stripped as above, before being returned to the same tank. For all sperm collected, sperm volume, and activity were determined.
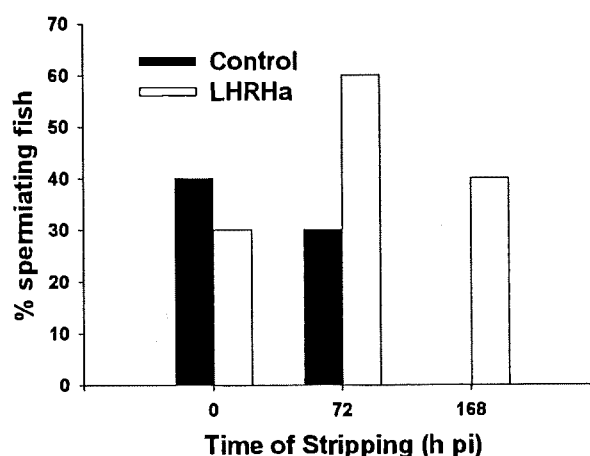
Figure A25: The proportion (%) of dhufish males spermiating 0, 72 and 168h following treatment with a slow release cholesterol pellet containing either 0 or 10 µg.kg^{-1} LHRHa. n= 10

Results/Discussion

At the first stripping, 40% of control-treated fish and 30% of LHRHa-treated fish could be stripped (Fig A25). By 72h post-treatment, the number of spermiating LHRHa-treated males had doubled to 60%, while in control fish it had decreased slightly to 30%. By 168h post-treatment, all control fish had ceased spermiating, while of the LHRHa-treated fish, 40% continued to spermiate.
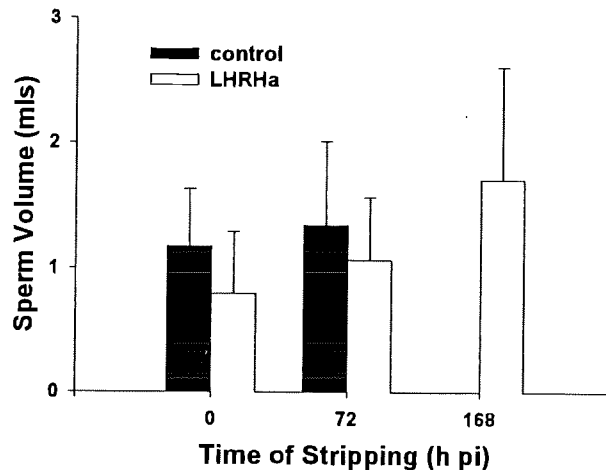
Figure A26: Volume of sperm stripped from dhufish males at 0, 72 and 168h following treatment with a slow release cholesterol pellet containing either 0 or 10 $\mu g.kg^{-1}$ LHRHa. n= 10. Values are means (excluding non-spermiating fish) ± SE.

At the time of treatment, both control and LHRHa-treated fish produced an average of between 0.75 and 1 ml of sperm (non-spermiating fish excluded; Fig A26). A similar volume of sperm was produced by 72h post-treatment in both treatments, with an average of around 1ml being produced in both control and LHRHa treatments. By 168h post-treatment, the mean volume of sperm stripped from LHRHa-treated males remained at greater than 1ml, while no sperm was
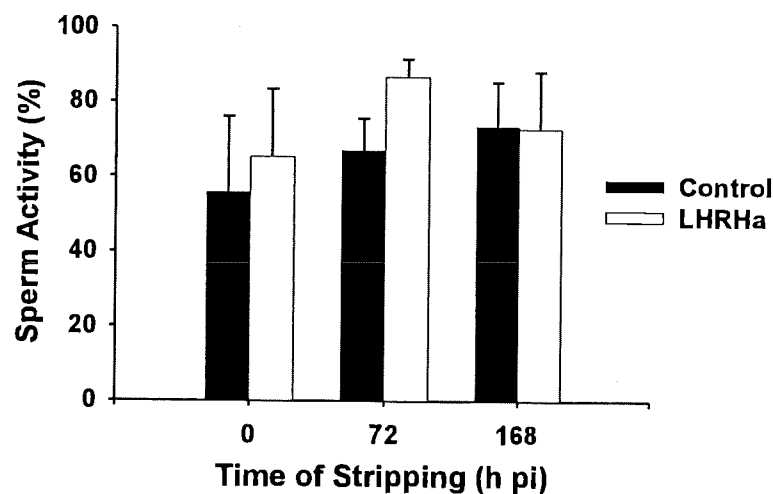
Figure A27: Activity of sperm stripped from dhufish males at 0,72 and 168h following treatment with a slow release cholesterol pellet containing 0 or 10ug.kg⁻¹ LHRHa.

produced by control fish. The mean volume of sperm stripped at 168h from LHRHa-treated fish was approximately twice that stripped at 0h, although not significantly different. The activity of sperm produced by fish in both treatment groups, and at each time was between 50 and 90% (Fig A27). There was no effect of treatment or on the timing of stripping.

Therefore, stress of repeated episodes of capture, handling and stripping caused a decrease in the number of fish producing sperm by 168 h post-implant. The volume of sperm from these fish was similar, as long as some fish were spermiating. Treatment with LHRHa at $10\mu g.kg^{-1}$ maintained spermiation with a similar percentage of fish spermiating at 0 and 168h post-treatment. The volume of sperm produced from LHRHa-treated fish appeared to increase from 0 to 168h, however, large variation precluded detection of significant differences. The activity of the sperm (used here as a measure of sperm quality) was not affected by stress, indicating the action of stress on male reproduction is on whether they spermiate or not, rather than sperm quality. Sperm activity, however, is a crude measure of sperm quality and so the effect of stress on fertilisation success of sperm and embryo quality still needs to be addressed. The use of LHRHa to maintain sperm production throughout the stress episode did not compromise activity of the sperm, adding support for its use to help overcome problems of sperm supply in captive dhufish.

Since conducting this experiment, problems have been experienced in the use of LHRHa to promote spermiation in captive dhufish. Although sperm activity is usually quite high (>70%) in fish stimulated by a LHRHa pellet, the activity rapidly deteriorates with time. "Natural" sperm can usually be stored a syringe for many hours, and often even for several days. "LHRHa-induced" sperm however is usually less than 10% active within a few hours. The longevity of sperm was not tested in the present study, however, this issue needs to be addressed before treatment with an LHRHa pellet can be recommended as a reliable means of securing sperm supply from captive dhufish.

Appendix K   Collection of sperm from different sources

Introduction

Sperm supply in dhufish limits the success of artificial propagation of the species. Captive male dhufish only produce a small amount of sperm compared to many other commonly cultured species. Recently techniques for cryopreserving dhufish sperm have been developed with some success. This technology, once refined, would enable a stock of cryopreserved sperm to be established for use when required. The limitation in sperm supply from captive males, however, means that there is little sperm available in the hatchery to conduct the research and in the longer term to establish a stock. Therefore, dhufish sperm will need to be obtained from fish from the wild. The simplest/cheapest way of obtaining sperm from wild-caught dhufish is to collect testes from fish at the fish market, prior to processing. Another source is to obtain fish from recreational fishermen. This study was conducted to compare the efficacy of sperm collected from fish at the fish markets and fish caught by recreational fishermen with sperm stripped from live captive male dhufish.

Methods

Sperm was collected from captive males at the ADU ("Captive"), fish from the fish market ("Market) or from fish from recreational angling competition ("Competition).

"Captive":   Fresh sperm was stripped from males being held at FMC and collected in a sterile syringe and stored on ice. Activity was measured by viewing sperm mixed with salt water under 40x magnification and determining the percentage of sperm moving. The time from stripping the sperm until measuring activity was less than 1h.

"Market":   Testes were collected from dead fish at the fish markets. The fish had been dead for up to 72 h and stored on ice. The testes were placed in a plastic bag and stored on ice for transport to the ADU where activity was measured as above. The time from removal of the testes until measurement of activity was less than 24h.

"Competition": Testes were collected from fish caught by recreational fishermen at a fishing competition. The testes were stored on ice and the activity was measured up on return to the ADU (as above). The time from removal of the testes until measuring activity was less than 5h.

Results/Discussion

Sperm stripped from captive males was significantly more active than sperm stripped from the testes of dead Competition and Market males (93%, 64% and 27% respectively; Figure A28). This difference is probably due to the delay from death until testing the activity of the sperm. Although the exact time is not known, this may have been as great as 97 h for Market fish, and

16 h for Competition fish. In contrast, for Captive fish there was a delay of less than 1 h. It is also possible, however, that the extra steps of removing the testes, and storing on ice might have affected the activity. Although not significantly different, the mean activity of sperm obtained from the Market fish was less than half that of sperm obtained from Competition fish (27 c.f. 64%). This again is likely to be due to the difference in time delay for Market and Competition fish.

These results suggest that the best source of sperm is live captive fish. It is likely that wild-caught fish stripped live, immediately following capture would yield similarly good sperm. For the purposes of cryopreservation it would be necessary to complete cryopreservation at sea to achieve optimal results. This would be logistically difficult but not impossible. In the absence of sperm stripped from live males, sperm stripped from the testes of males soon after death would be a suitable alternative. In the present study, testes were removed from the fish and stored in plastic bags on ice. There is anecdotal evidence to suggest that leaving the testes inside the dead fish, and storing the whole fish on ice may better preserve the sperm quality.

Time Delay (h)

Time from death until removal of testes	<72	<10	na
Time from removal of testes to measuring activity	<24	<5	na
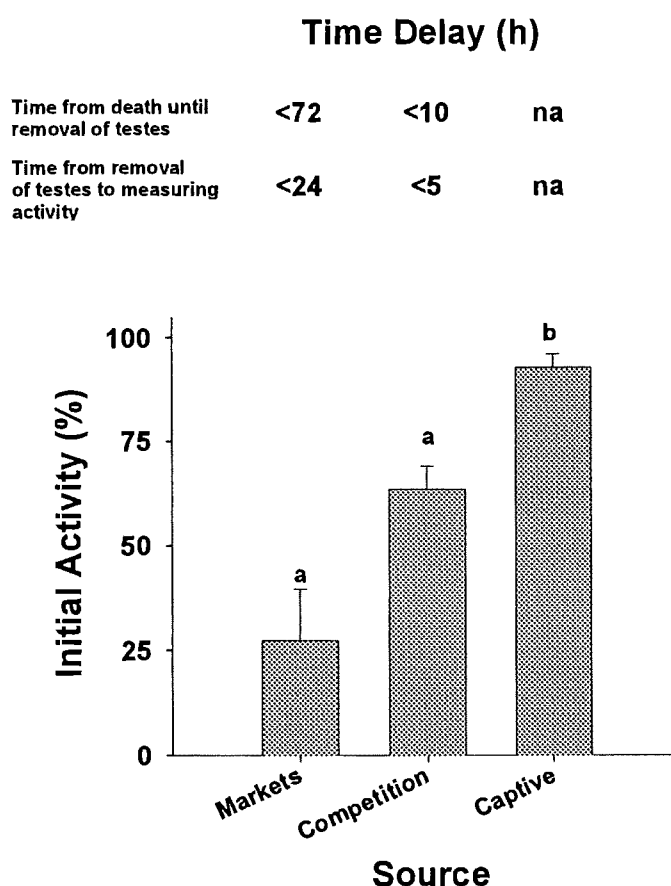
Figure A28: Activity of sperm, collected from a variety of sources: 1) testes removed at fish markets (Markets; n=9); 2) testes removed following an angling competition (Competition; n=17); and 3) sperm stripped from live fish (Captive, n=9). Values are means + SE. Different letters show significantly different Tukey groupings among sources.

Appendix L   Cryopreservation of Dhufish Spermatozoa

Author: Tahryn Mackrill

This report was written in partial fulfilment of the Industry Practicum component of the Bachelor Degree of Marine Science, School of Environmental Science, Murdoch University

ABSTRACT

The dhufish, *Glaucosoma hebraicum*, is a highly valued fish species, with great potential in aquaculture. Many problems are associated with the breeding of this species in captivity, including particularly low semen availability compared to other species. The cryopreservation of dhufish semen is a technique with potential to overcome this problem.

Semen from dhufish was preserved using two different diluents: 1) Ringers, DMSO and glycerol, and 2) Ringers and glycerol. This was to determine if the two diluents would have a significant impact on the outcome of cryopreservation. The effect of these diluents on dhufish spermatozoa was not detrimental, although the rate of diluent addition was found to influence sperm activity.

Diluted semen was frozen in 1.2mL cryogenic vials or in 0.25mL straws to determine if the storage vessels would influence the success of cryopreservation. The cryogenic vials resulted in significantly higher activities than the straws.

Overall, a fast freeze rate was compared to a slow freeze rate to determine if this would affect the outcome of cryopreservation. It was evident that a fast freeze rate resulted in higher activities than the slow freeze rate.

To compare the technique for cryopreserving spermatozoa, black bream (*Acanthopagrus butcheri*) and pink snapper (*Pagrus auratus*) trials were also conducted. The snapper spermatozoa were more robust than both black bream and dhufish spermatozoa.

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Firstly and foremost, I would like to thank my industry supervisor, Dr Jennifer Cleary, for her endless patience, willing assistance, encouragement and friendship throughout the course of this project. Without her knowledge and guidance, I would have been lost.

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A big thankyou to the members of the MAAC and the Busselton Fishing and Angling Club, for allowing me to collect my fish samples and set up my endless amount of equipment.

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Finally, thankyou to my family and friends, for showing interest, support and understanding.

Introduction

The Dhufish, *Glaucosoma hebraicum* (of the family Glaucosomatidae), is endemic species to the south west of Australia. It has a limited distribution from Koks Island at Shark Bay, southwards to Cape Leeuwin (McKay 1997). They are carnivorous, bottom dwelling fish found mostly in reefs submerged under 30 meters or more of water (Abord 1986, Ashby 1996). In the wild, dhufish have a very slow growth rate, however, they can grow up to and over 27 kg (Hesp and Potter 2000).

Little is known of the biology of dhufish, and few studies have been conducted on the species. Dhufish are multiple spawners (Hesp and Potter, 2000). Their natural spawning season in the wild is between late December and April (Hesp and Potter, 2000) providing a very limited time frame for studies on reproductive biology to be conducted.

Due to its high value, the dhufish is subject to considerable fishing pressure both recreationally and commercially. The production of dhufish stocks through aquaculture is an alternative way of fulfilling commercial requirements, thereby reducing the pressure on the wild fish population. Aquaculture of dhufish also provides the possibility of restocking cultured dhufish into the oceans.

Breeding dhufish through aquaculture has proved to be a difficult task, and for many reasons. Primarily, female dhufish caught from the wild will not ovulate or spawn naturally when held in captivity. The Aquaculture Development Unit (ADU) of the Fremantle Maritime Centre are currently running a dhufish breeding program. They are hopeful that, provided conditions are optimal, the female fish bred at the centre (now 4 years of age) will begin to spawn without assistance in their broodstock tanks. This is an ongoing trial. In the absence of naturally spawning fish, hormone treatment is required. Whilst administering specific hormone doses will evoke ovulation, spawning still does not normally occur and the eggs must be manually stripped and fertilised (Cleary 1998). Work done by Dr Jennifer Cleary at the ADU has focused specifically on this problem. To begin the process of stripping and fertilisation, the eggs within the female fish must be released from the ovary into the oviduct (ie. ovulated). In order to do so, the female is treated with the hormone LHRHa (luteinising hormone releasing hormone analogue) or HCG (human chorionic gonadotropin) to induce ovulation. Once ovulation has occurred, the female is anaesthetised so that the eggs within her can be stripped manually. Semen is collected from male dhufish in a similar fashion, and is then added to the freshly stripped eggs and gently mixed. Salt water is added to activate the sperm cells and begin the process of fertilisation. With the initial problems relating to egg production overcome, the successive problems of sperm production arise.

Availability of good quality dhufish semen is often an impediment to the successful production of fertilised eggs. Male dhufish held in captivity will spermiate naturally if their time in the tank has

been sufficient for them to acclimatise to the environment (Cleary 1998). However, it has become apparent that without hormonal assistance only the dominant male in a tank (ie, the healthiest and fittest - usually the largest, unless it is ill) will produce semen. Further problems arise with the volume of semen produced by each male. A large male dhufish of approximately 9 kg will yield less than 1 mL of semen on any one day. In contrast, a small pink snapper of approximately 2 kg can yield up to 10 mL of excellent quality semen. This is directly related to testes size. A dhufish testes constitutes only 0.1% of the total body weight as opposed to 5-10% of the body weight in a snapper (Hesp and Potter, 2000).

Although hormone induced males produce larger volumes of semen, and with more than one male in a tank spermiating at a time, the semen produced tends to be less viscous than naturally produced semen. The sperm cells do not appear to remain active for a long enough period of time, suggesting that they are of poorer quality. Further studies will be conducted in the future in an attempt to refine the hormone induction technique. However, due to the small volume of semen available in wild-caught fish, it is unlikely that hormone therapy will completely overcome limitations in semen supply.

Cryopreservation is a technique that could help to overcome the problems associated with limited semen supplies. Cryopreservation is essentially the "long-term preservation of biological material in a frozen but viable form at ultra-low temperatures, usually below -130°C" (Bromage and Roberts 1995). Presently, any attempts to cryopreserve fish ova have been unsuccessful.

In contrast, spermatozoa have been successfully cryopreserved for many years. The origin of spermatozoan cryopreservation dates back to 1953, with Blaxter's work on herring spermatozoa. Since then, cryopreservation has developed and is now a well-established procedure. Many problems are associated with cryopreserving sperm cells, although for many of the species of fish examined, these problems have been overcome. The preservation of spermatozoa provides a great number of benefits to aquaculture. In the case of dhufish, cryopreservation technology could be used to overcome limitations in semen supply by establishing a 'sperm bank'. This would guarantee a supply to meet fertilisation requirements.

Cryopreservation, although successful in many species, has never been established for the dhufish. The fact that limited studies on any aspect of this species have been conducted makes work on them quite difficult. In previous years at the ADU, trials to cryopreserve dhufish sperm were undertaken. Success was achieved in terms of freezing and thawing the sperm, although fertilisation of eggs with the thawed sperm was unsuccessful (Cleary, Jenkins and Partridge 1999). The limited success is not suprising, considering that there are a number of factors that may affect the success of cryopreservation.

As a result, this project was conducted in an attempt to determine a successful method for cryopreserving dhufish spermatozoa. Through experimentation, the factors affecting cryopreservation of dhufish semen were explored. However, the pre-mentioned problems

associated with low semen availability for this species placed limitations on the study. Additional trials were conducted on black bream (*Acanthopagrus butcheri*) and pink snapper (*Pagrus auratus*) to determine if the efficacy of the cryopreservation technique varies between species.

The determination of an effective method for cryopreservation involved testing whether the use of different diluents and storage vessels would significantly impact the success of freezing. Two diluents were compared, one containing the cryoprotectants DMSO and glycerol, and one containing simply glycerol. The two storage vessels compared were 1.2mL sterile cryogenic vials and 0.25mL cryogenic straws.

Materials and Methods

Dhufish - Trial 1

Collection of Semen

Semen was stripped from the testes of wild caught dhufish on the 8th January 2000, at the Marmion Angling and Aquatic Club (MAAC) fishing competition. The testes were removed from male dhufish several hours after death. The time of removal, length of the fish, and whether or not the fish was kept chilled (ie. on ice) were recorded. Once removed, the testes were placed in sealed plastic bags, and stored on ice until needed.

After making a small section in the testes with a scalpel, a pasteur pipette was used to take a small sample of the semen to determine the activity of the sperm. This was called the initial activity. Once an initial activity reading was taken from each sample, the scalpel was used to cut open the testes at one end. Starting from the anterior end and gently working down with smooth fauceps (or fingers), the semen was stripped out and into labelled 2mL vials. The maximum volume of semen allowed in each vial was 0.4 mL (to allow for dilution - see below). Thus, multiple vials were used for each testes. The vials were kept in a stand within an ice bath so as to keep the samples continuously chilled.

Determining activity

A small sample of semen was taken using a pasteur pipette, and smeared onto a glass microscope slide. A glass cover slip was placed over the smear, and the slide was examined under a compound microscope. At this point, the sample should have been inactive, indicating that no seawater had contaminated the semen. If the sperm cells were already activated, the sample could not be cryopreserved and so no further processing was conducted. If the sample was inactive, an activity reading was taken.

To the slide containing the inactive sperm cells, a drop of seawater was added by gently lifting the cover slip, and replacing it after addition. With the seawater added, the live spermatozoa activate and will be seen swimming rapidly on the microscope slide. Any dead sperm cells can be seen as immobile dots. By examining the slide on a magnification of 40x, an approximation of the number of active cells amongst inactive cells in the field of view was taken. This was repeated at different positions on the slide to gain an approximation of the percentage of active sperm cells for the entire sample. Any sperm cells vibrating on the spot, but not swimming were not counted as active spermatozoa. Sperm activity was used to measure the quality of the semen.

Processing of semen – Comparison of DMSO vs glycerol

Once the semen was separated into vials, the diluents were added. Two diluents were trialed (Table A 15): 1) Marine Teleost Ringers (Table A16) with glycerol and 2) Marine Teleost Ringers with glycerol and dimethyl-sulphoxide (this diluent is referred to as DMSO). Approximately half of the vials from any one fish were allocated to the DMSO treatment, and the remainder to glycerol.

Table A15: **Composition of the two diluents used for diluting semen samples prior to cryopreservation.**

	Diluent	
	DMSO	Glycerol
Marine Teleost Ringers	50 mL	50 mL
Glycerol (5%)	2.5 mL	2.5 mL
DMSO (6.25%)	3.25 mL	N/A

The diluent was added in four equal portions using an automatic pipette. Each portion was equivalent to the volume of semen, so that the final ratio of semen to diluent was 1:4. The rate of diluent addition was quite rapid for this trial. During dilution, the vials were kept on ice. Between each diluent addition, the vial was gently shaken to mix the diluent with the semen. Once diluted, a small sample was taken from each vial and the activity of the sperm cells was recorded (as above). This was called the pre-freeze activity.

Table A16:   Composition of the Marine Teleost Ringers used in both diluents. Adapted from   Palmer, Hogan and Barlow, 1994.

Component	50mL Stock Solutions (in 50mL dist H_2O)	Ringers Solution (vol of stock)	Final Concentration of Ringers (g/L)
NaCl	No stock	7.25 g	7.25
KCl	3.8 g	5 mL	0.38
$CaCl_2.2H_2O$	2.4 g	5 mL	0.24
$MgSO_4.7H_2O$	2.7 g	5 mL	0.27
$NaHCO_3$	1 g	0.5 mL	0.01
$NaH_2PO_4.2H_2O$	4.1 g	5 mL	0.41
D+ glucose	10.1 g	5 mL	1.01
Distilled water		975.5 mL	

Cryopreservation of semen – Comparison of straws vs vials

Two storage vessels were used: straws and vials. The diluted semen was portioned into cryogenic straws and sterile cryogenic vials to prepare for freeze. The semen was drawn into the 0.25 mL straws using a syringe with fine tubing on the end which attached firmly to the top of the straw. An automatic pipette was used to place 100 µL samples of diluted semen into the1.2mL cryogenic vials. Approximately half of the semen diluted in glycerol was stored in straws, and the other half was stored in vials. Semen diluted with DMSO was treated this way also.

Vials and straws were taped onto the end of a thin piece of PVC rod. A 1.5 L thermos flask was half-filled with liquid nitrogen. The samples were secured in the neck of the flask by a clamping the rod to a retort stand (Fig A29). Gradually, the rod was lowered into the liquid. An electronic thermometer was also attached to the rod, to monitor the temperature change. On average, a freeze rate of 3 to 5 $^\circ$C.min^{-1} was applied.

Once the temperature reached -50 $^\circ$C, the samples were completely submerged in the liquid. They were removed from the rod and transferred to the liquid nitrogen dewar for storage (the straws were in goblets attached to the canes, and the vials were attached directly onto the cane). They were stored in the dewar for no particular length of time, until the thawing of samples was conducted.
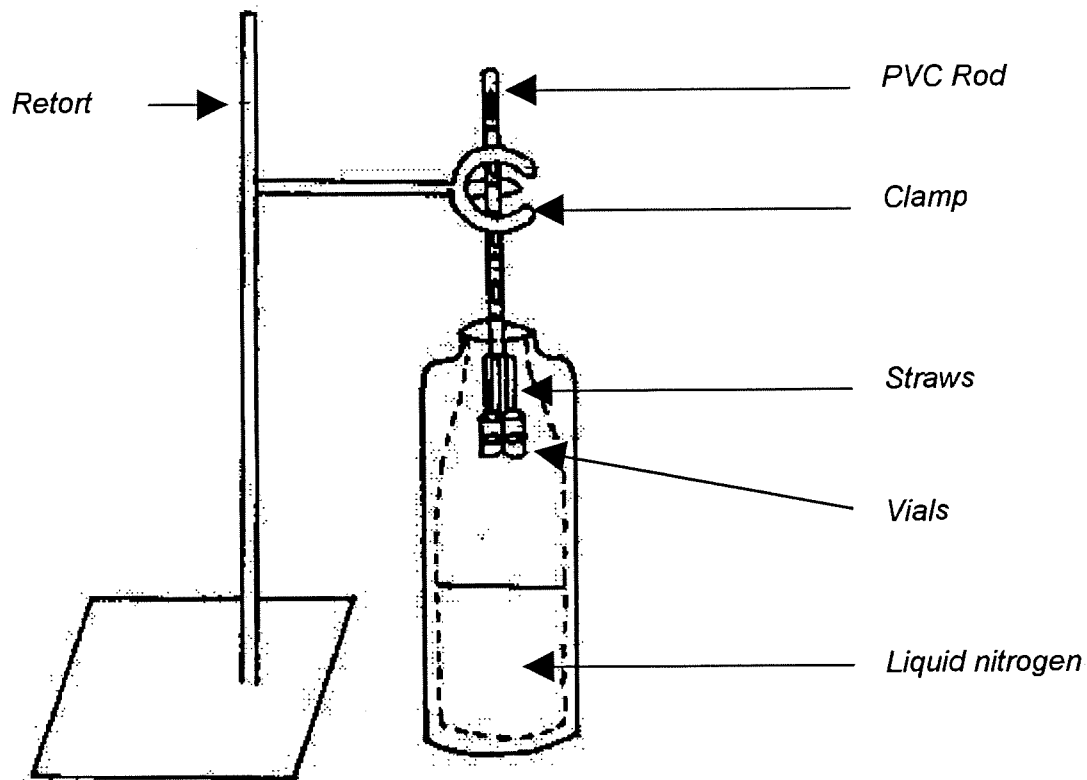
Figure A29: Set up of equipment used in dhufish trial 1. This was a slow freeze in liquid nitrogen vapour.

Thawing of semen

The straws and vials were thawed at a range of different temperatures in an attempt to find the most successful. Thus, cold water baths as low as 20 °C and hot water baths up to 50 °C were used. Some samples were thawed at room temperature. The temperature used was recorded for each sample.

Once thawed, a slide was prepared from each sample to obtain the final activity reading for the trial. This was the post-freeze activity.

Dhufish - Trials 2 and 3

Collection of semen

Trial 2 – Wild-caught

This trial was also conducted at the MAAC, on the 5th February 2000 at the fishing competition. The method of collecting the semen was identical to the method used in dhufish trial 1.

Trial 3 - Captive

Unlike the first two dhufish trials, which collected semen from dead dhufish, the third trial was conducted on live fish. The semen was collected opportunistically (whenever tanks were drained) from the captive male dhufish held in the broodstock tanks at the ADU. The fish were anaesthetised using 2-phenoxyethanol, at a dose rate of 0.3 ml/L. Once anaesthetised, the fish was lifted out of the tank, rinsed thoroughly with freshwater on its abdominal area and gently towelled dry. This prevents contamination of semen with salt water and anaesthetic. By gently applying pressure along the abdomen from behind the pectoral fins, toward the vent, the semen was forced out of the vent. It was collected in 1mL or 2mL sterile syringes.
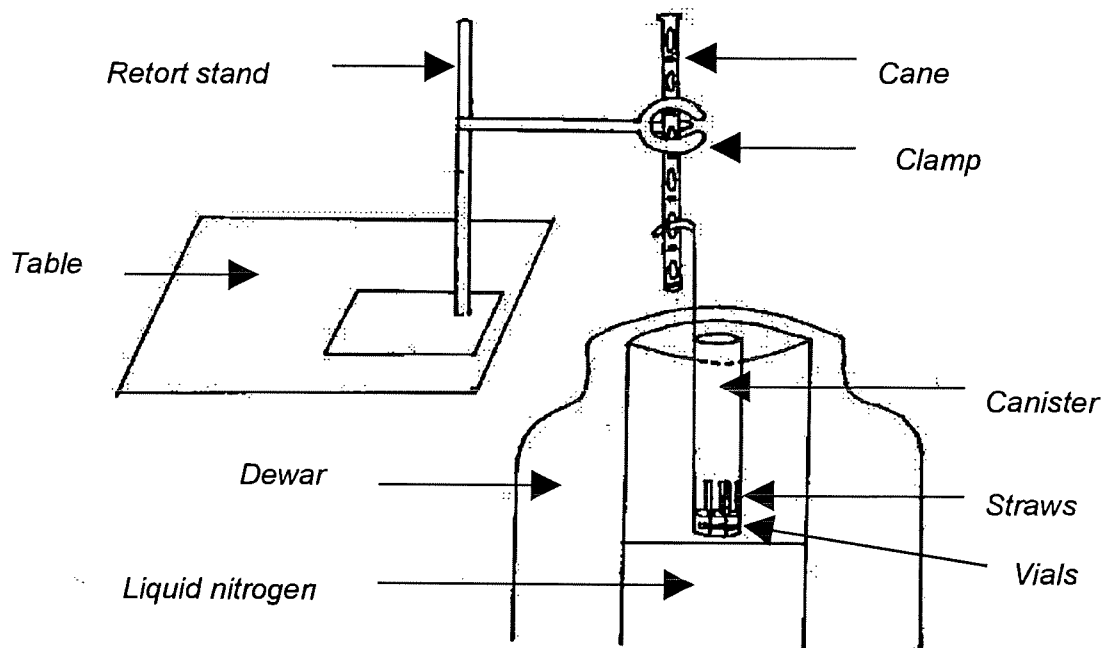
Figure A30: Set up of equipment used for dhufish trials 2 and 3, all black bream trials and the snapper trial. This was a rapid freeze in liquid nitrogen vapour.

Processing of semen – Comparison of DMSO vs glycerol

The processing of semen for both trials was almost identical to the processing in dhufish trial 1. The only variation was the rate of dilution. The addition of diluent to the semen in trial 2 was slower than for the first trial, yet no set time was allowed between each addition. However, in trial 3, the addition of diluent to the semen samples allowed a 30 second interval between successive additions.
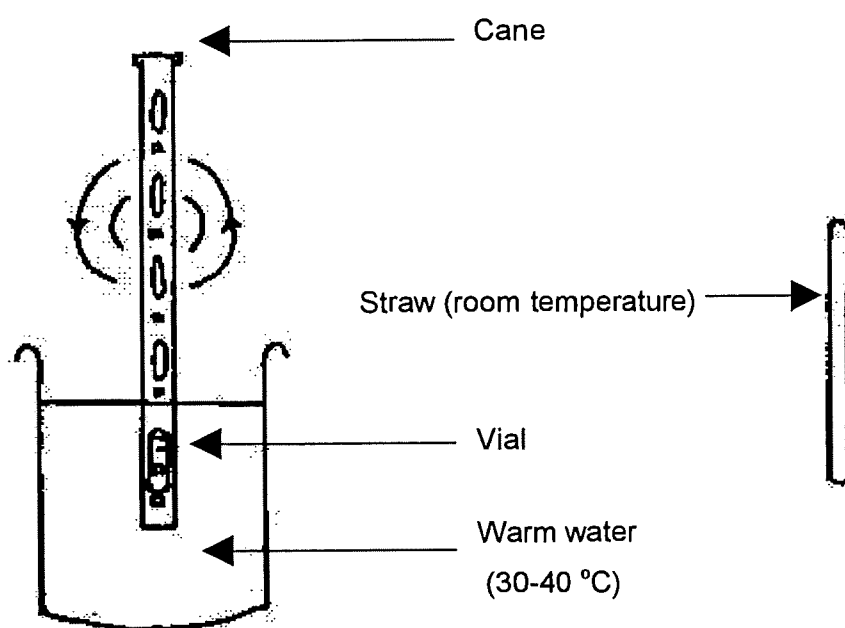
Figure A31: **Thawing of vials and straws for dhufish trials 2 and 3, all black bream trials and the snapper trial.**

Cryopreservation of semen – Comparison of straws vs vials

The portioning of samples from the vials into the straws and cryogenic vials was identical to the procedure in dhufish trial 1. However, the rate of freezing the samples was more rapid.

Rather than a slow freeze, the method chosen for cryopreserving was a rapid freeze in the liquid nitrogen vapour. A canister from the dewar was removed, and positioned within the dewar so that the base was held just above the surface level of the liquid nitrogen. The canister was kept in this place by hooking the handle through a cane held by the clamp on a retort stand (refer to Fig A30). A few vials and straws were then simply dropped into the canister, and left for 10 minutes to ensure that the freeze was complete. Due to limitations in equipment sensitivity, the rate of freeze for this method is unknown, yet the electronic thermometer indicated a drop of -50°C in less than 30 seconds.

Thawing of semen

Rather than thawing over a range of temperatures as in dhufish trial 1, the straws and cryogenic vials in these trials were thawed at set temperatures recommended by Leung (1983). The straws were thawed for 3 minutes at room temperature. The vials were swirled in a warm water bath between 30 and 40 °C for 1.5 minutes (Fig A31).

Black Bream - Trials 1-4 and Snapper

Collection of semen

The black bream and snapper semen for these trials was collected from live fish held in tanks at the ADU. The method of collection was the same as that described for dhufish trial 3.

Processing of semen – Comparison of DMSO vs glycerol

The processing of the semen was similar to dhufish trials 2 and 3, with 30 second intervals between successive diluent additions.

Cryopreservation of semen – Comparison of straws vs vials

The method of freezing the samples was the same as that described for dhufish trials 2 and 3 - a rapid freeze in liquid nitrogen vapour.

Thawing of semen

The thawing for all of these trials was the same as in dhufish trials 2 and 3.

Table A17 : Summary of the varying techniques used for collecting, preparing, cryopreserving and thawing semen samples for dhufish, black bream and snapper trials.

	Source	Dilution	Diluents	Freeze Rate	Thaw Rate	
					Straws	Vials
Dhufish Trial 1	Dead	Fast	DMSO & Glycerol	Fast	Varied room-50 °C	Varied room-50 °C
Dhufish Trial 2	Dead	Med.	DMSO & Glycerol	Slow	Room	30-40 °C
Dhufish Trial 3	Alive	Slow	DMSO & Glycerol	Slow	Room	30-40 °C
Black bream Trials 1-4	Alive	Slow	DMSO & Glycerol	Slow	Room	30-40 °C
Snapper Trial 1	Alive	Slow	DMSO & Glycerol	Slow	Room	30-40 °C

Analysis of data

For each species, the raw data was sorted into each of the four treatments being tested - DMSO with straws, DMSO with vials, glycerol with straws and glycerol with vials. For each of these treatments, the mean, standard deviation and standard error was calculated, using the average activities for initial, pre-freeze and post-freeze.

Statistics were used to make comparisons between these treatments. For these analyses, dhufish trials 2 and 3 were pooled together, due to their similar techniques. The subsequent increase in sample size increased statistical power. The same was done for all of the black bream trials, which were pooled together into one trial.

Data was analysed by ANOVA. Prior to performing ANOVA, homogeneity of variance was tested using Bartlett's test. If the variance was homogenous, no further transformations were required prior to further analysis. To test differences between diluents and vessels, a two-way ANOVA was performed on each data set (initial, pre-freeze and post-freeze activity). If no significant differences occurred between the diluents or between the vessels, and there was no interaction, the analysis was complete. If one significant difference occurred, a one-way ANOVA (t-test) was performed by pooling the data, disregarding the insignificant component. If two significant differences occurred, a comparison of means was conducted using the Tukey-Kramer HSD test. All analysis was conducted using the statistics computer package, JMP Version 3.1.1.

The Tukey-Kramer HSD test was then used to conduct a comparison of means for the mean initial, pre-freeze and post-freeze activities from each species. This test indicated any significant differences within species between each of these activities. It also indicated if any relationships between species existed. Dhufish trials 2 and 3 were treated as different trials for this analysis, in order to visualise if any difference occurred between the wild-caught and the captive dhufish. Due to the large sample size of the black bream trial (1-4 combined) compared to the other trials, 50 data entries were randomly removed prior to analysis. This created approximately equal sample sizes for each species, and increased to chance of obtaining equal variances.

RESULTS

Dhufish - Trial 1

The mean activity decreased during dilution, with initial activities of about 75% decreasing to 50-60% after dilution. The comparison of means from this trial (Fig A32) indicated that this difference was significant. The decrease during the freeze was greater, with mean post-freeze activities ranging from 0-25% only. This decrease from pre-freeze activity was significant (Fig A33).

After the addition of the diluent, the glycerol samples had significantly higher activities than the DMSO samples (P=0.02; Fig A33). A significant difference was also indicated between the vessels. However, Bartlett's test on the one-way ANOVA indicated that variances were not homogenous. An attempt to arc-sin transform the data was made, yet the variances remained unequal. The figures in Table A18 below explain this.

Table A18 :   The range of post-freeze activities for dhufish trial 1.

Activity Range for Post-Freeze	Frequency
0-10%	36
10-30%	1
30-50%	6
50-70%	1
70-90%	1

With the bulk of the activities occurring within the narrow range of 0-10%, the curve for the distribution of this data is not 'normal'. Since the data does not have a normal distribution, no valid analyses can be performed. This is not important however. The importance lies in the fact that the majority of samples had a post-freeze activity close to, or equal to, zero.

Dhufish - Trials 2 and 3 combined

In the second trial, a significant decrease in activity occurred after the addition of diluent to the samples. This decrease occurred during the freeze also, with post-freeze activities significantly different to pre-freeze activities (Fig A34). The mean post-freeze activity for this trial was significantly higher than for trial 1.
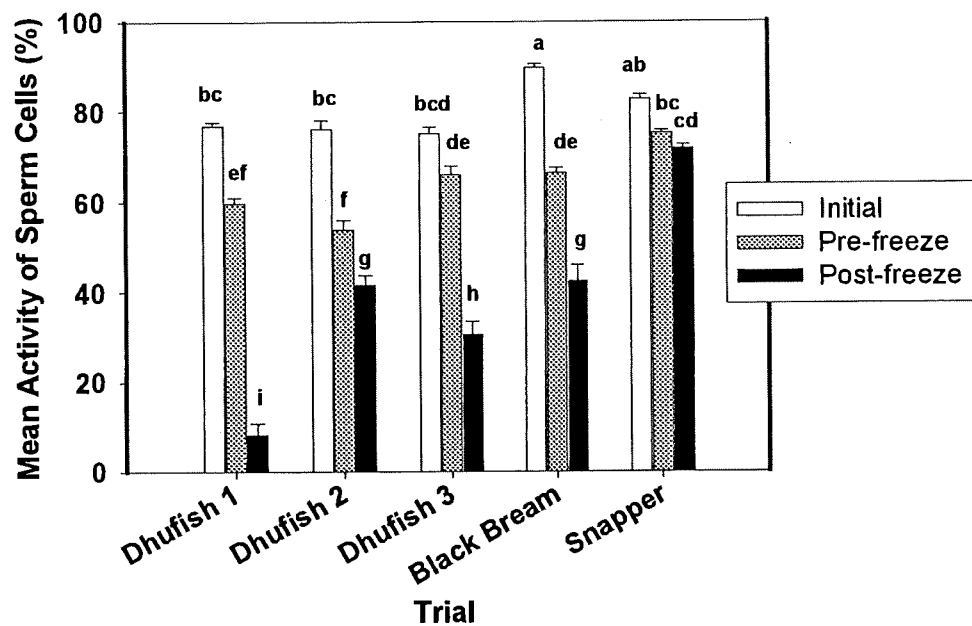
Figure A32: Comparison of means for initial, pre-freeze and post-freeze activities for each dhufish, black bream and snapper. Different letters indicate significantly different Tukey-Kramer HSD grouping (P<0.01).

The comparison of means for the third trial indicated that the dilution had no significant effect on the pre-freeze activity. However, the decrease from pre-freeze to post-freeze activity was significant (Fig A32). This post-freeze activity is significantly higher that the first trial, yet significantly lower than the second.

The combined data from dhufish trials 2 and 3 indicated that the activities of DMSO samples were higher than that of glycerol samples (Fig A34). The difference between these two diluents was significant (P=0.0137). A significant difference also occurred between the vessels after freezing. Once thawed, the activities of the vials were significantly higher than the activities of the straws (P=0.0263; Fig A34).

Figure A33: Mean initial, pre-freeze and post-freeze activities of the sperm cells for each treatment within Dhufish trial 1. The scatter plot illustrates a significant difference between diluents for the pre-freeze activity.

Figure 34: Mean initial, pre-freeze and post-freeze activities of the sperm cells for each treatment within Dhufish trials 2 and 3. The scatter plots illustrate significant differences between diluents for the pre-freeze activity, and storage vessels for the post-freeze activity.

Black Bream - Trials 1-4 combined

The mean activity of the sperm cells after dilution was significantly lower than the initial activity (Fig A32). The decrease during freeze was also significant, with pre-freeze activities of above 63% dropping to 30-55% at post-freeze. The mean post-freeze activity was the same as the post-freeze activities in dhufish trial 2.

The two diluents did not result significantly result in different activities after dilution. The comparison of means for the different treatments at post-freeze (Fig A35) illustrates that vials were significantly higher than straws with DMSO as a diluent. Yet, they are not significantly different with glycerol.

Snapper - Trial 1

The comparison of means for this trial indicated that no significant decreases in activity resulted from dilution or during the freeze (Fig A32). However, a significant decrease in initial activity to post-freeze activity was apparent.

The only significant difference that occurred between treatments was between the two diluents. DMSO samples had significantly higher activities than glycerol samples after dilution (P<0.0001; Fig A36).

Comparison between species

The mean initial activities for the dhufish trials and the snapper trial were the same. The black bream trial however, had a mean initial activity significantly higher than all of the dhufish trials (Fig A32).

The mean pre-freeze activities for the snapper trial and dhufish trial 3 were not significantly affected by the addition of diluents. However, dhufish trials 1 and 2 and the black bream trial showed a significant decrease in activity when diluents were added.

The mean post-freeze activity for the snapper trial was significantly higher than for all other trials. The mean post-freeze activities for dhufish trial 2 and the black bream trial were the same. Dhufish trial 1 had a dramatically low post-freeze activity, which was significantly different from all the others.
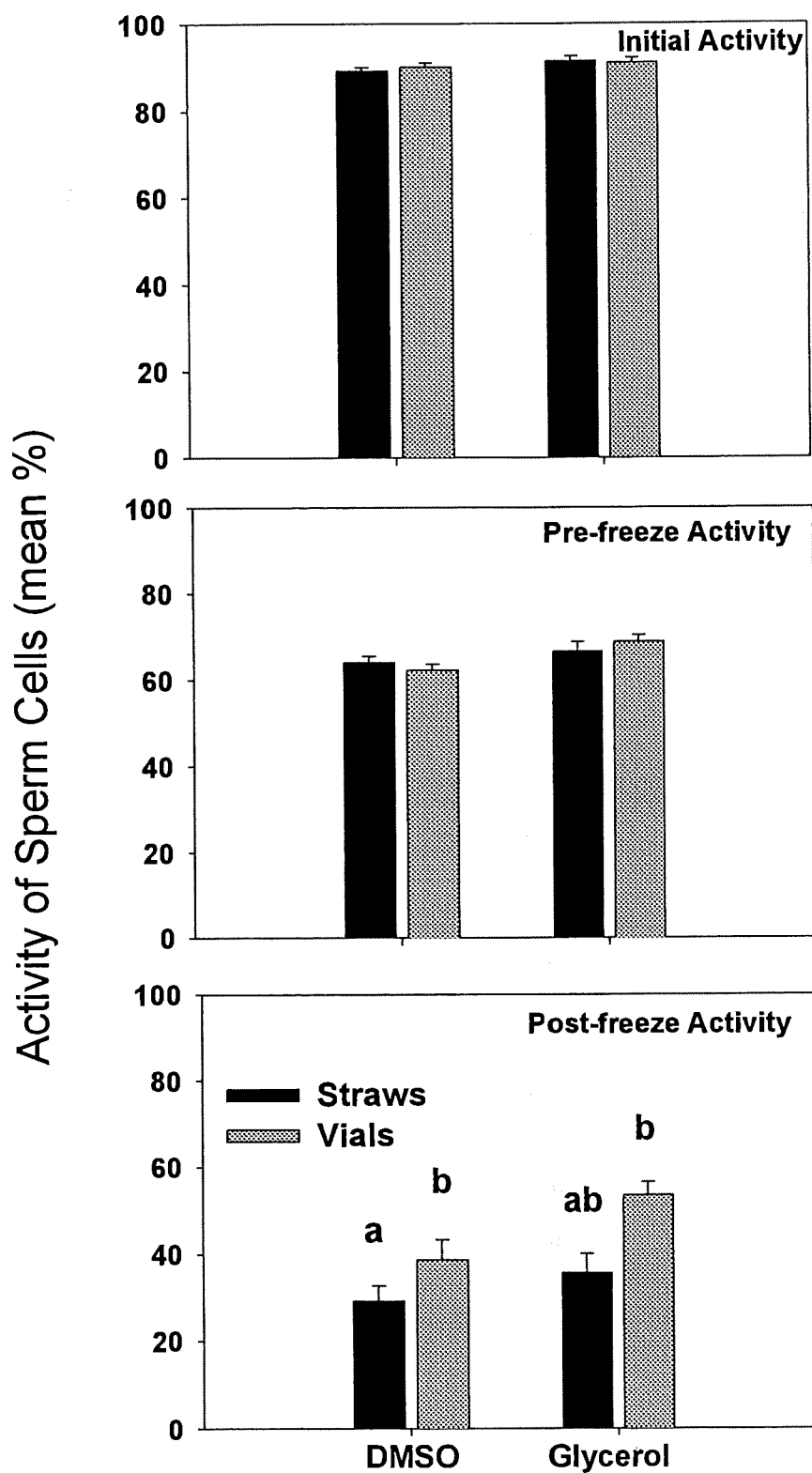
Figure A35: Mean initial, pre-freeze and post-freeze activities of the sperm cells for each treatment within the black bream trial. The different letters on the post-freeze activity graph indicate significant differences.

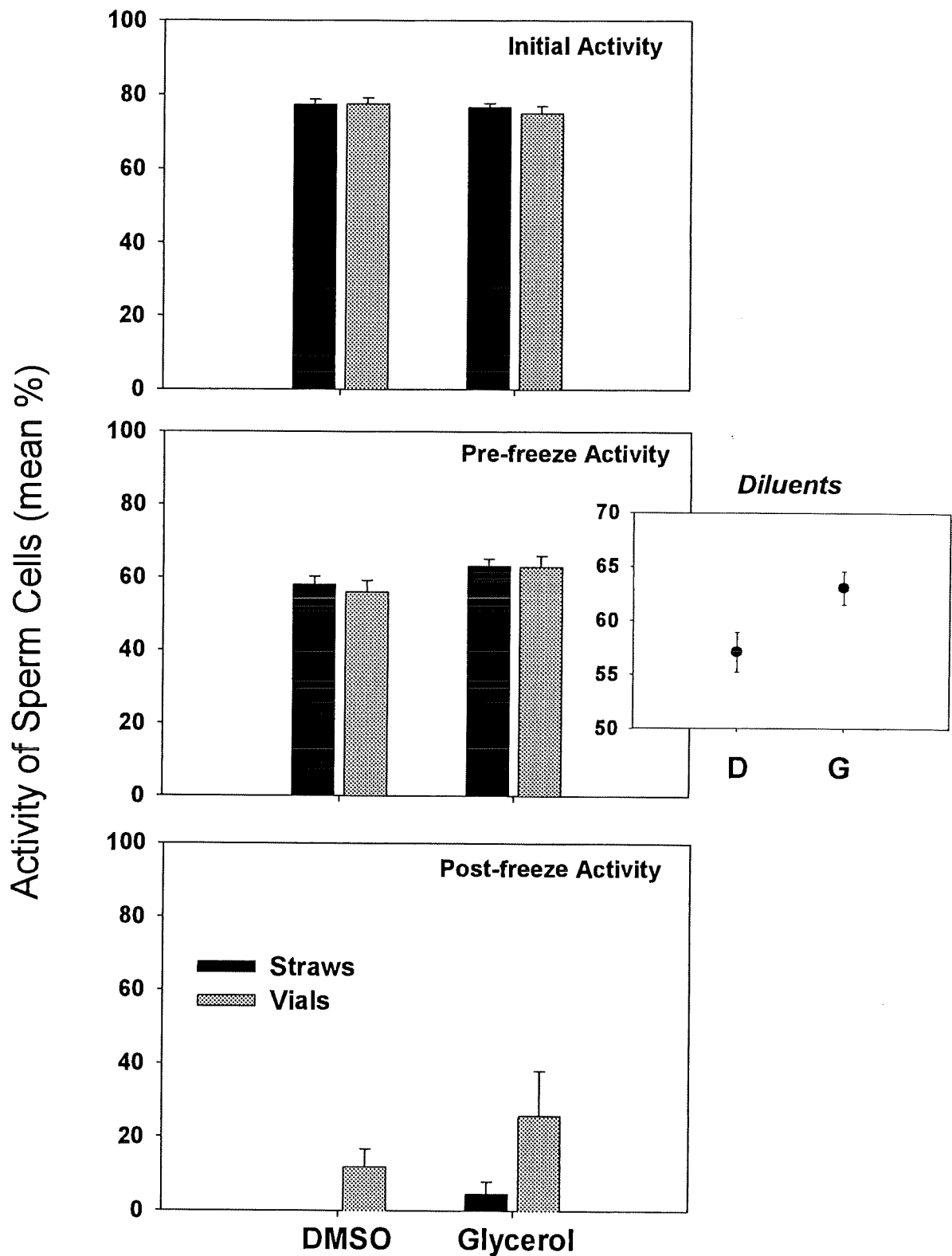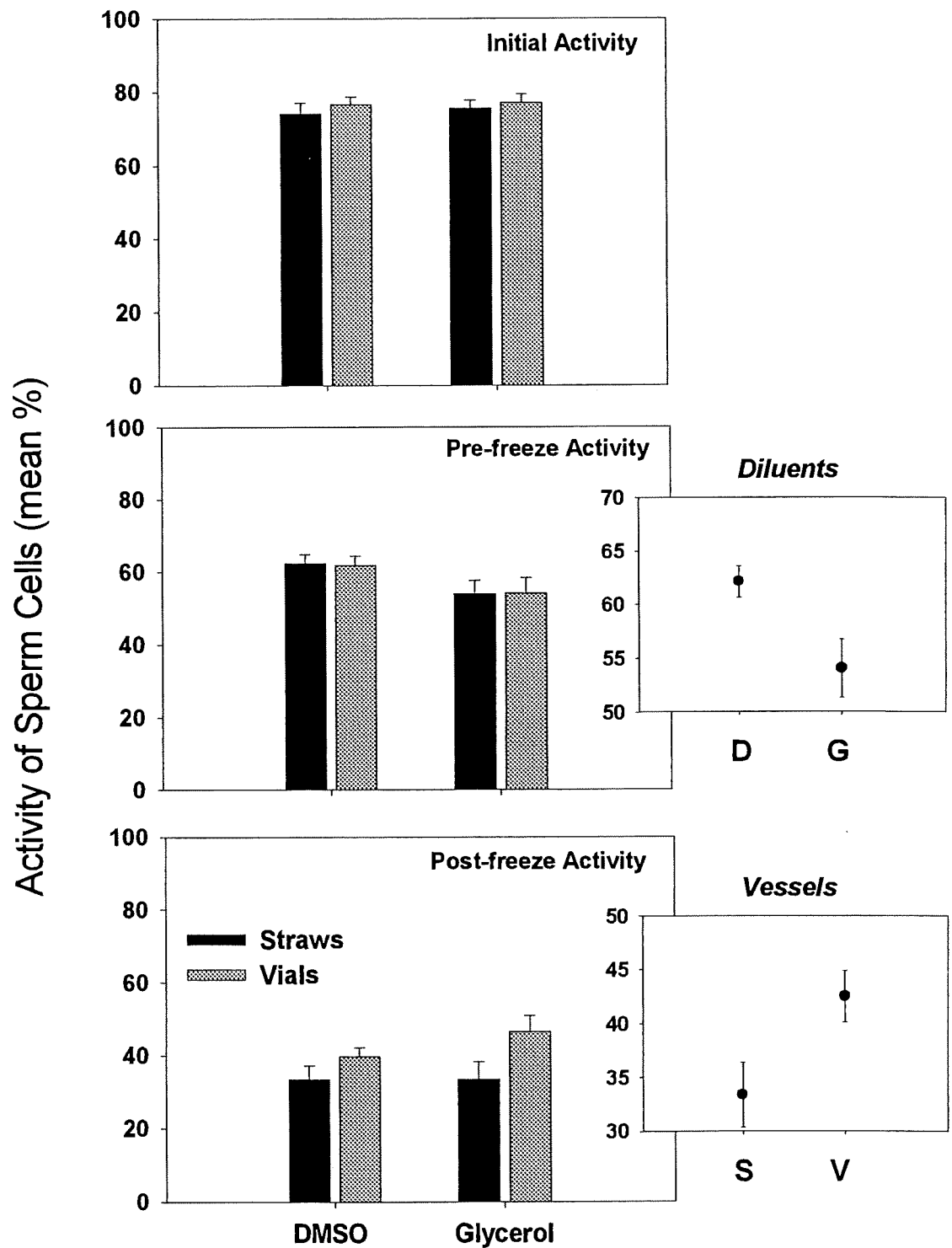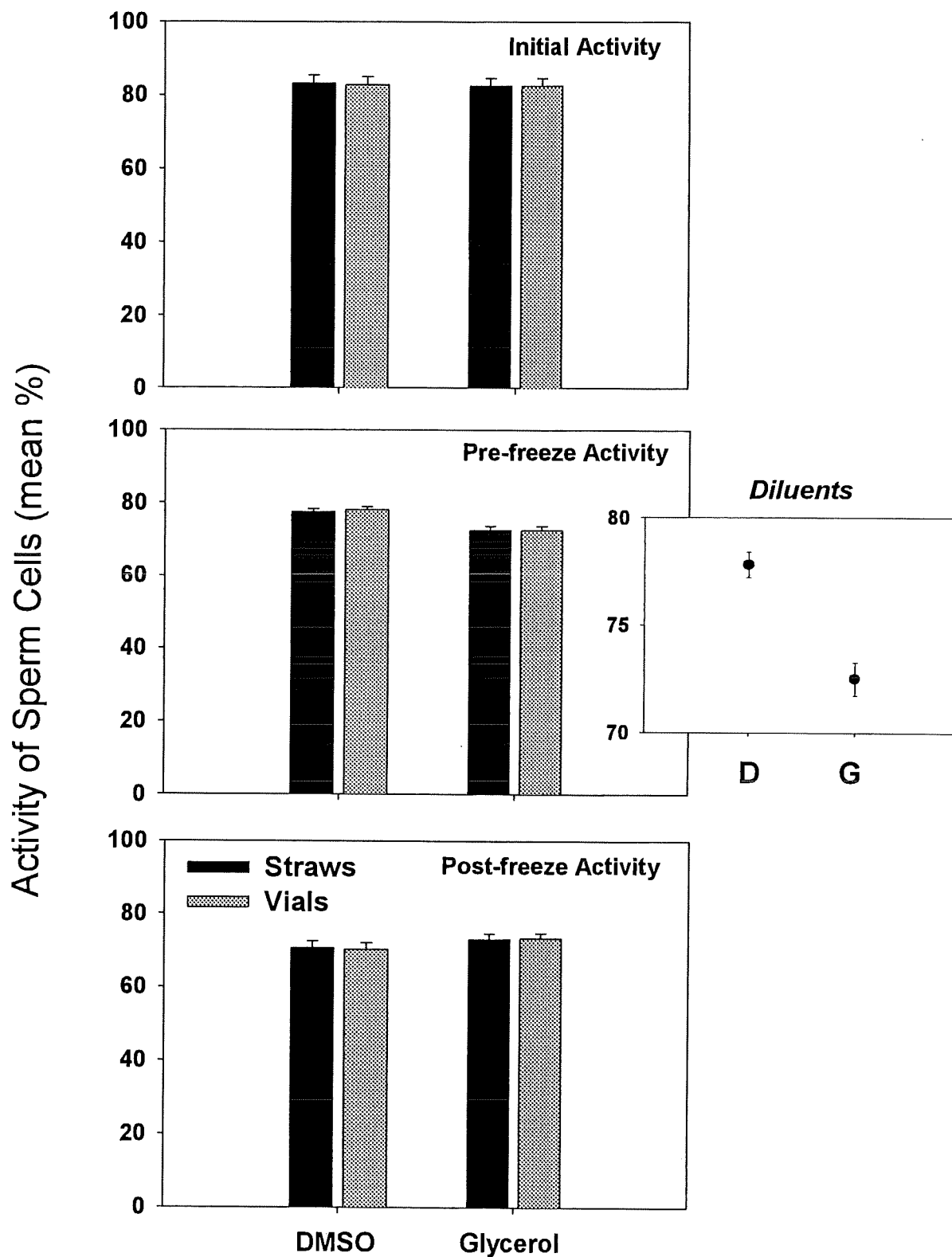**Figure A36:** Mean initial, pre-freeze and post-freeze activities of the sperm cells for each treatment within the snapper trial. The scatter plot illustrates a significant difference between diluents for the pre-freeze activity.

DISCUSSION

Dilution

Osmotic shock

The dramatic decrease in activity that occurred after dilution from initial activity to pre-freeze activity in all trials (except the snapper) can be partially attributed to osmotic shock. The diluents added to the semen samples both contained Marine Teleost Ringer solutions, as well as either DMSO or glycerol, which function as cryoprotectants. If these solutions are added to sperm cells too rapidly, the sudden change in ionic composition of the extracellular solution surrounding the cells creates great osmotic pressure. Following the laws of osmosis, water will either be lost from the sperm cells if the extracellular solution is higher in ionic concentration, or drawn into the sperm cells if the extracellular solution is lower in ionic concentration. Osmotic shock would result with rapid dilutions, as the great osmotic pressure created so suddenly would not allow adequate time for the gradual movement of water into or out of the sperm cells (Palmer, Hogan and Barlow 1994).

This would explain why a significant decrease in activity resulted after the addition of diluents in dhufish trials 1 and 2, but not in trial 3. The longer, 30 second intervals between each successive addition of diluent in the third trial would have minimised the effect of osmotic shock. The snapper activities were not affected by diluent addition, so osmotic shock is not an issue. However, the black bream activities decreased significantly after dilution. Since diluent addition was fairly slow throughout the trials, it is possible that osmotic shock is not the only issue.

Toxicity of cryoprotectants

Another factor that could be contributing to reduced activities after diluent addition is related directly to the cryoprotectants. Various cryoprotectants become toxic to biological material at high concentrations (Leung 1987). DMSO is known to be one of the most toxic cryoprotectants (Leung 1987, Bromage and Roberts 1995, Stoss and Holtz 1983). However, when used at the correct concentration, it can also be one of the most successful. Likewise, glycerol has been known to be toxic to salmonid spermatozoa (Gwo *et al* 1991, Scott and Baynes 1980, Stoss 1983). Yet, studies on yellowfin bream concluded that glycerol was the most successful cryoprotectant (Thorogood and Blackshaw 1992). The use of a cryoprotectant is species specific, and so one will not be universally successful for all species.

It is therefore possible that one of the cryoprotectants used in the dhufish, black bream and snapper trials may have been slightly toxic to one or more of these species. This should be considered for the dhufish and black bream trials in particular, where the activity of sperm cells was significantly reduced after the addition of diluents. In dhufish trial 1 glycerol performed better than DMSO. In contrast, the combined data from the second and third trials indicated that

DMSO out-performed glycerol. Given this apparent conflict, it is unlikely that either diluent is more toxic to the dhufish spermatozoa. Thus, the reduction in activity after dilution of dhufish spermatozoa is more likely a result of the above-mentioned osmotic shock, than a result of toxic cryoprotectants. Either way, it becomes apparent that dhufish spermatozoa are quite sensitive to changes in their extracellular medium.

The black bream spermatozoa appear to be just as sensitive to such changes. With no significant differences between DMSO and glycerol for pre-freeze activity, it appears that neither cryoprotectant is more toxic to the sperm cells. However, it is possible that both diluents are toxic to black bream, and maybe even dhufish, spermatozoa. The DMSO diluent and the glycerol diluent both contain glycerol. Subsequently, if glycerol is toxic to the black bream sperm cells, both diluents would have detrimental effects on activity after addition. Further studies need to be conducted to determine is glycerol is toxic to black bream and dhufish spermatozoa.

The snapper spermatozoa appear to be highly resistant to change in their extracellular surroundings. The addition of diluents to the semen samples did not result in a significant decrease in activity as with the other two species. Rather, the pre-freeze activity remained fairly high, suggesting that snapper sperm cells are quite tolerant and hardy. However, for pre-freeze activity, the DMSO was significantly higher than the glycerol. This could be due to a slight toxic effect from the glycerol, yet since both diluents contain glycerol, this is not very likely. The difference could probably be attributed to sampling error.

Cooling and Thawing rates

A significant decrease in activity from pre-freeze to post-freeze occurred for all of the trials, except for snapper. The decrease in activity after thawing can be attributed to a number of factors, including the freeze and thaw rates. The most consequential factor influencing success after cryopreservation is the rate of freeze. As a solution of semen and diluent is cooled, the water molecules surrounding the sperm cells begin to freeze and the concentration of the extracellular solution subsequently increases. With a slow cooling rate, there is ample time for the movement of water, and so water is lost from the spermatozoa in an attempt to maintain osmotic equilibrium with the extracellular solution (Leung 1987). This results in the dehydration of spermatozoa, which can be fatal depending on the size of the cells and the actual cooling rate (Bromage and Roberts 1995). The slower the cooling rate, the more severe the dehydration and the less likely that the spermatozoa will survive. In contrast, with a high cooling rate the movement of water is prohibited by the shorter time, and dehydration does not occur. However, insufficient time for water to diffuse out of the spermatozoa leads to freezing within the cells, again as an attempt to equilibrate with the extracellular solution. This is known as intracellular ice (Leung 1987), and will result in rupture of the sperm cell membrane. Obviously, a balance needs to be met to ensure success in freezing spermatozoa. The correct freeze rate for fish spermatozoa is very species specific.

The rate of freeze, however, is not the only factor influencing the outcome of cryopreservation. The thawing rate is also a crucial factor. With a slow thaw rate, small ice crystals left within the sperm cells after freeze will reform into larger ice crystals as they reabsorb the volume of water lost during cooling (Leung 1987). This process is called recrystallisation, and results in the formation of damaging intracellular ice. Therefore, high thawing rates are more successful, as they allow insufficient time for the absorption of water and subsequent recrystallisation.

The first dhufish trial illustrates superbly the outcome of cryopreserving dhufish spermatozoa with a slow freeze. With a freeze rate averaging only -3 to -5 °C a minute, it is assumed that there was adequate time for the total dehydration of the sperm cells. This would explain the high frequency of dead samples after thawing, indicating that this cryopreservation procedure was simply unsuccessful. The second and third dhufish trials were more successful. As the major difference in technique between these two trials and the first one was the more rapid freeze rate, the difference can be partially attributed to the use of this freeze rate rather than a slow one.

The thaw rates used for the first dhufish trial ranged from room temperature to 50 °C. With such a range of temperatures, and still no successful post-freeze activities, it is emphasised that the slow freeze rate was solely responsible. After the completion of this first trial, it was opted to follow Leung's guidelines for thawing straws and vials (1987). In all other subsequent trials, straws were thawed at room temperature and vials at 30-40 °C. For dhufish trials 2 and 3, and the black bream trials, these temperatures appeared to result in some success.

However, the overall results from these trials were not optimal, with post-freeze activities significantly lower than the initial readings. Ideally, the post-freeze activity should be similar, or equal, to the activity of the sperm cells initially. Thus, it is indicated that the freeze and thaw rates were not optimal. Since these rates are species specific, further investigation is required to find the correct freeze and thaw rates for cryopreserving dhufish and black bream spermatozoa..

The results from the snapper trial were somewhat more successful than for the dhufish and black bream. With no significant decrease from pre-freeze to post-freeze activities, it is assumed that the cooling rate and thawing rate were more efficient on snapper spermatozoa than the other species. However, a significant difference in activity from initial to post-freeze still remains. Thus, although the freeze and thaw rates were satisfactory, they were not optimal.

The straws and vials used as vessels for the semen samples seemed to have a small impact on the success of cryopreserving dhufish and black bream spermatozoa. Vials were significantly higher in activity for dhufish trials 2 and 3 combined, and for the combined black bream trials with DMSO as the diluent. In the first dhufish trial, this pattern was also suggested, yet unequal variances can not allow this to be conclusive.

Overall, the pattern appears to suggest that vials will provide slightly more successful post-freeze activities than straws for these species. In contrast, the snapper spermatozoa were not

significantly affected by the storage vessel. The vessel used for freezing appears to be species specific, as with most of the factors influencing success after cryopreservation. For instance, 0.25mL straws used for cryopreserving salmonid spermatozoa proved more successful than pellets (Erdahl *et al* 1984). In contrast, studies on the Atlantic halibut concluded that no significant difference occurred between samples frozen in straws or as pellets (Bolla *et al* 1987).

It is therefore possible that vials are significantly more successful for cryopreserving black bream and dhufish spermatozoa, yet not snapper spermatozoa. However, the results gained from this project are not conclusive, and further studies need to be carried out.

Conclusion

Cryopreservation is an extremely complex process. Several problems are associated with cryopreserving dhufish, black bream and snapper spermatozoa. Firstly, a dilution effect was prevalent for the dhufish and black bream trials. With rapid additions of diluent, osmotic shock causes harm to sperm cells and decreases activity. For the black bream spermatozoa, it also appeared that the two diluents both had a slight toxic effect. It is suggested that this was due to the presence of glycerol in both. The snapper spermatozoa did not seem to suffer from dilution effects at all.

Secondly, a consistent decrease during freeze for the dhufish and black bream indicated that the freeze and thaw rates were not optimal for these species. It was apparent, however, that a fast freeze rate is more favourable to dhufish spermatozoa than a slow one. The faster rate prevents the dehydration of sperm cells during freeze. The freeze and thaw rates used throughout the trials produced satisfactory results for the snapper spermatozoa, yet they were not optimal.

The results also suggested that vials are more effective at freezing dhufish and black bream spermatozoa than straws. However, snapper spermatozoa were not affected.

All of these factors are species specific, indicating that successfully cryopreserving dhufish semen is a difficult task. The work done in this project has merely provided a beginning for the studies that need to be conducted to discover a successful freeze method for dhufish spermatozoa. However, knowing that dilution effects and freeze/thaw rates have a significant impact on semen from this species, refining the method for cryopreservation should continue from here.

If the breeding of dhufish in captivity is to be a long-term aquaculture project, then further refinement of a cryopreservation method should seriously be considered. Continued limitation in semen availability is an impediment to the successful breeding of dhufish, and cryopreservation could overcome this problem.

REFERENCES:

Abord, J., 1986. The reproductive biology of the Westralian Jewfish, *Glaucosoma hebraicum* (Pisces:Glaucosomatidae). Part 1. Gonad morphology. Biology project 301/302. Curtin University, WA.

Ashby, D., 1996. Decompression sickness in Westralian Jewfish (*Glaucosoma hebraicum*). Faculty of Agriculture, University of Western Australia.

Blaxter, J.H.S., 1953. Sperm storage and cross-fertilisation of spring and autumn spawning herring. Nature (London), 172: 1189-1190

Bolla, S., Holmefjord, I. and Reftsie, T., 1987. Cryogenic preservation of Altantic halibut sperm. Aquaculture, 65: 371-374

Bromage, N.R. and Roberts, R.J., 1995. Broodstock management and egg and larval quality. Blackwell Scientific.

Cleary, J.J., 1998. Report to Fisheries Research and Development Corporation. Progress to 30 June 1998. Aquaculture Development Unit, Fremantle Maritime Centre, South Metropolitan College of TAFE.

Cleary, J.J., Jenkins, G.I. and Partridge, G.J., 1999. Preliminary manual for the hatchery production of WA dhufish (*Glaucosoma hebraicum*). Aquaculture Development Unit, Fremantle Maritime Centre, South Metropolitan College of TAFE.

Erdahl, A.W., Erdahl, D.A. and Graham, E.F., 1984. Some factors affecting the preservation of salmonid spermatozoa. Aquaculture, 43: 341-350

Gwo, J., Strawn, K., Longnecker, M.T. and Arnold, C.R., 1991. Cryopreservation of Atlantic croaker spermatozoa. Aquaculture, 94: 355-375.

Hesp, S.A. and Potter, I.C. 2000. Determination of the biological parameters required for managing the fishery of West Australian dhufish. FRDC Project 96/103. Murdoch University, Murdoch WA.

Leung, K.P.L., 1987. Fish spermatology. Ultrastructure, phylogeny and cryopreservation. University of Queensland.

McKay, R.J., 1997. FAO species catalogue. Pearl perches of the world (Family Glaucosomatidae). An annotated and illustrated catalogue of the pearl perches known to date. FAO Fisheries Synopsis. 125(17). Food and Agriculture Organisation of the United Nations, Rome, 26p.

Palmer, P.J., Hogan, A.E. and Barlow, C.G., 1994. Chilled storage of pikey bream (*Acanthopagrus berda*) sperm and activation in different salinities. Asian Fisheries Science, 7: 35-40

Scott, A.P. and Baynes, S.M., 1980. A review of the biology, handling and storage of salmonid spermatozoa. J.Fish Biol., 17: 707-739

Stoss, J., 1983. Fish gamete preservation and spermatozoa physiology. In: W.S. Hoar, D.J. Randall and E.M. Donaldson (Editors), Fish Physiology, Vol 9B, Academic Press, New York, NY.

Stoss, J. and Holtz, W., 1983. Cryopreservation of rainbow trout (*Salmo gairdneri*) sperm. IV. The effect of DMSO concentration and equilibrium time on sperm survival, sucrose and KCl as extender components and the osmolality of the thawing solution. Aquaculture, 32: 321 - 330.

Thorogood, J. and Blackshaw, A., 1992. Factors affecting the activation, motility and cryopreservation of the spermatozoa of the yellowfin bream, *Acanthopagrus australis* (Gunther). Aquaculture and Fisheries Management, 23: 337-344.

Hormone-induced spawning and development of artificially reared larvae of the West Australian dhufish, *Glaucosoma hebraicum* (Glaucosomatidae)

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Abstract.   Hormone-induced spawning of the West Australian dhufish, *Glaucosoma hebraicum* (Glaucosomatidae) is reported, and the artificially-reared eggs and larvae are described. Hormones tested were hCG, Ovaprim and LHRH analogue, the last both as implants and in a saline solution. Late-stage fertilized eggs are spherical (960–1200 μm), possess a pigmented oil globule (210–273 μm) anteriorly on the unsegmented yolk, and hatch in about 32 hours at ~23°C. Newly hatched larvae (1.75–2.10 mm) have a prominent yolk sac which is resorbed after 3.3 days. The mouth becomes functional from Day 3, first feeding occurs from Day 3.5 and the inflated gas bladder is first observed from Day 4.3. Larvae examined (1.75–8.13 mm) have a moderately deep body, 24–25 myomeres, early-forming pelvic fins that are heavily pigmented by the postflexion stage, small to moderate head spination, no gap between anus and anal-fin origin, and a distinct pattern of pigmentation. Notochord flexion occurs between 3.42 (15 days) and 4.83 mm (25 days), and the transition to the juvenile stage starts from ~8 mm (45–46 days). Comparisons with taxa with similar larvae show that *Glaucosoma* larvae share some similarities with pempherid larvae, although the evidence is not sufficient to strengthen the view that both groups are closely related.

Extra keywords: eggs, spawning induction

Introduction

The West Australian (WA) dhufish, *Glaucosoma hebraicum* Richardson, 1845 (Family Glaucosomatidae) is predominantly a benthic species endemic to Western Australia, commonly found around caves and ledges on offshore reefs between Shark Bay (25°30'S,114°30'E) and the Recherche Archipelago (33°52'S,121°54'E), to depths of ~200 m (Hutchins and Swainston 1986; Paxton and Hanley 1989; McKay 1997). The dhufish is a highly regarded table fish and one of the most popular recreational fish in Western Australia, attaining a maximum size of 1.22 m total length and 26 kg (Hutchins and Swainston 1986; Kailola *et al.* 1993; McKay 1997). It is caught throughout the year across most of its distributional range, with commercial catches of 100–200 t year^{-1} between 1992–93 and 1996–97 (Kailola *et al.* 1993; Anon. 1997).

A great deal of knowledge of early life history of temperate Australian fishes has been gained from fish culture. Whereas aquaculture research on freshwater species has been aimed mainly at rearing larvae for restocking programmes in inland waterways (e.g. Rowland 1983, 1988), rearing of marine and estuarine fishes has been aimed at species suitable for commercial aquaculture (e.g. Ruwald *et al.* 1991; Battaglene and Talbot 1992, 1994. In Western Australia there has been success in rearing black bream (*Acanthopagrus butcheri*) and snapper (*Pagrus auratus*)

and, in the past two years, the WA dhufish *G. hebraicum* (Jenkins and Frankish 1997). Biological data on dhufish suggest that this species may have attributes that make it highly attractive for aquaculture, including fast initial growth and easy handling in captivity (Hesp 1997). In addition, a market survey suggested that an opportunity existed in Australia for its commercial aquaculture (I. Barnetson, unpublished).

The limited information on the reproductive biology of *G. hebraicum* indicates that this species is a multiple spawner, spawning at depths of 40–50 m between late November and April, with a peak in late January and early February. In addition, fecundity estimates range from 0.3 million to 4 million eggs, with females reaching first maturity at 25–30 mm standard length by the end of their fifth year (Cockman and McGuire 1986; Hesp 1997). Despite several ichthyo-plankton surveys carried out in waters south of Shark Bay (WA) in recent years (e.g. Jonker 1993; Kendrick 1993), neither eggs nor larvae of *G. hebraicum* have been reported, and no records are available of juveniles <60 mm total length (Sudmeyer *et al.* 1990). The present paper briefly describes the hormone-induced spawning of *G. hebraicum* and provides a description of the early life-history stages of this species based on material reared in the laboratory. The methods and results presented here on the hormone-induced spawning are only preliminary, and controlled quantitative

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studies are required. The descriptions of the eggs, and of the larvae from the yolk-sac through to the postflexion stage, constitute the first for fishes of the family Glaucosomatidae.

Materials and methods

Broodstock

Glaucosoma hebraicum adults (1.6–12.0 kg) were caught by hand-line between 1995 and 1997 off Lancelin (31°0'S,115°18'E), in depths of <25 m. Upon capture, fish were placed directly into a 250-L seawater container and transported to the research centre for acclimatization. Captive dhufish were kept in several fibreglass and concrete 10–50 m³ tanks at densities of 0.5 to 3.0 kg fish m⁻³ with continuously flowing seawater, and a minimum exchange of 10% h⁻¹. Male:female ratio ranged from 1:1 to 1–2:3–4. A net (500 μm mesh) was installed on each tank overflow to collect naturally spawned eggs.

Hormonal treatment

Broodstock fish were anaesthetized with 300 mL m⁻³ of 2-phenoxy-ethanol. Each fish was tagged intraperitoneally with a passive transponder 11.5 mm long × 2.0 mm diameter. For examination of maturity stage, sperm were sampled by manual stripping and oocytes by using a sterile plastic cannula. Oocytes were examined under a dissecting microscope and the diameters (μm) of the largest oocytes were measured with an eyepiece micrometer.

Males. A total of 17 males were treated with human chorionic gonadotrophin (hCG), and LHRH analogue (LHRH-a; des-Gly¹⁰,[D-Ala⁶]-luteinizing hormone-releasing hormone ethylamide) in a 95% cholesterol-base slow-release implant (LHRH-a *imp*) and in a saline solution (Table 1).

Females. A total of 22 females with oocytes ≥400 μm in diameter were injected intraperitoneally with various dosages of hCG, LHRH-a *imp*, LHRH-a in a saline solution, and Ovaprim containing gonadotrophin-releasing hormone (GnRH) and Domperidone (Table 1). The sterile needles used for injection were 18-gauge for liquid hormones and 2 mm diameter for implants. Fish were anaesthetized and their eggs were stripped manually at the first sign of ovulation.

Artificial fertilization

Sperm samples were collected in sterile syringes by manually stripping anaesthetized fish. Each sample was examined under a compound microscope and sperm showing low or no motility was rejected. The selected sperm was kept at room temperature for a maximum of 1 h before it was used.

Eggs from females that had ovulated were manually stripped into plastic containers, transferred into a 1-L container of seawater (filtered to 1 μm absolute) and sterilized by ultraviolet radiation. Sperm from at least two males was added to the suspension of eggs, gently mixed and left for about

5 min, and the floating fraction then disinfected and transferred into an incubator. Fertilized eggs were rinsed with 22–24°C seawater (filtered to 1 μm absolute), sterilized by ultraviolet radiation and incubated

Rearing of larvae

Larvae were reared under continuously flowing seawater that was filtered to 1 μm absolute by cartridge filtration and sterilized by ultraviolet radiation. Water temperature was maintained between 22° and 24°C. Larvae were fed rotifers (*Brachionus plicatilis*) and developmental stages of brine shrimp (*Artemia salina*).

Material examined

Naturally and hormonally induced spawned eggs of *G. hebraicum*, both unfertilized and fertilized, were measured to the nearest 10 μm under a dissecting microscope fitted with an eyepiece micrometer (Table 2). A total of 131 laboratory-reared larvae (1.75–8.13 mm body length, BL) were used (Table 3) to describe morphometrics and pigmentation. Larvae had been fixed in 5% formaldehyde and later preserved in 70% ethanol. Representatives of the larvae examined were lodged with the Australian Museum (Sydney). Two artificially reared juveniles (22.54–24.25 mm BL) were also examined (excluded from Table 3).

Terminology used to describe head spines and morphometric measurements of larvae follows Neira *et al.* (1998). All measurements were made to the nearest 0.01 millimetre under a dissecting microscope fitted with an eyepiece micrometer. Body length (BL) corresponds to notochord length (tip of snout to tip of notochord) in preflexion and flexion larvae, and to standard length (tip of snout to posterior margin of hypurals) in postflexion larvae. Measurements of body depth (BD), head length (HL) and preanal length (PAL) given throughout the text and in Table 3 are expressed as a percentage of body length. Body depth and head length (in terms of % BL) were used to describe a larva as either very elongate (BD <10%), elongate (BD 10–20%), moderate (BD 21–40%) or deep (BD 41–70%), and as having a small (HL <20%), moderate (HL 20–33%) or large head (HL >33%); preanal length was used to characterize a larva as having a moderate (PAL 30–50%), long (PAL 51–70%) or very long gut (PAL >70%). Pigment described refers solely to melanin. Illustrations were made with the aid of a camera lucida.

Results

Natural spawning

The smallest captive male and female dhufish in spawning condition were 47 cm total length (2.2 kg) and 43 cm total length (1.6 kg), respectively. Natural maturation in males was recorded between December and April. Most females underwent gonadal development but did not ovulate or spawn naturally.

Induced spawning

Induced maturation was initially best achieved with implants of about 100 μg kg⁻¹ LHRH-a *imp*. Saline injections of hCG and LHRH-a did not induce spawning in males, but LHRH-a *imp* stimulated some sperm production. Sperm produced by males injected with LHRH-a *imp* was similar to that obtained in mature males that were not injected, and contained a high density of highly motile spermatozoa. The three hormones tested on females (Table 1) induced final maturation of oocytes and ovulation, particularly LHRH-a which induced egg production at all dosages. However, although treatments with either LHRH-a

Table 1. Hormone treatments in male and female *Glaucosoma hebraicum*

	Hormone	Dosage
Males	hCG	770–1130 IU kg⁻¹
	LHRH-a *imp*	29–111 μg kg⁻¹
	LHRH-a (saline solution)	67–80 μg kg⁻¹
Females	hCG	870–1200 IU kg⁻¹
	LHRH-a *imp*	5–125 μg kg⁻¹
	LHRH-a (saline solution)	5 μg kg⁻¹
	Ovaprim	6 μg GnRH kg⁻¹

Table 2. Diameter (μm) of eggs of *Glaucosoma hebraicum* spawned naturally and after hormone treatment
n, no. of eggs measured. F, fertilized; UF, unfertilized

	Naturally spawned UF	870 IU kg^{-1} UF	hCG 1200 IU kg^{-1} UF	1200 IU kg^{-1} F	LHRH-a *imp* 100 μg kg^{-1} UF	109 μg kg^{-1} F
Range	1000–1200	1000–1120	960–1060	1000–1080	1000–1060	1000–1100
Mean ± 2 s.e.	1074 ± 21	1047 ± 24	1014 ± 7	1041 ± 15	1035 ± 10	1046 ± 5
n	20	14	51	14	17	76

Table 3. Body length, age and body proportions of larval stages of
Glaucosoma hebraicum reared in the laboratory
n, no. larvae; BL, body length; HL, head length; BD, body depth; PAL,
preanal length. Values for HL, BD and PAL given as percentage of body
length and shown as range and mean ± 2 s.e.

	Yolk sac (*n* = 36)	Larval stage Preflexion (*n* = 47)	Flexion (*n* = 23)	Postflexion (*n* = 25)
BL (mm)	1.75–2.60	2.30–3.95	3.42–4.80	4.83–8.13
Age (days)	0–3	3–15	16–27	25–46
HL (% BL)	9.2–25.0 (16.3 ±1.6)	18.8–39.1 (29.0 ± 1.5)	34.9–50.0 (40.8 ± 1.5)	35.2–59.1 (43.1 ± 1.8)
BD (% BL)	7.8–21.7 (13.0 ± 1.3)	16.5–27.3 (20.0 ± 0.7)	33.5–42.4 (37.9 ± 1.0)	36.8–50.7 (44.8 ± 1.4)
PAL (% BL)	48.9–65.4 (56.2 ± 1.4)	46.1–66.5 (54.1 ± 1.2)	59.3–68.7 (64.0 ± 0.9)	64.1–73.5 (68.3 ± 1.0)

Fig. 1. Body length (mm) v. age (days) of reared larvae of *Glaucosoma hebraicum*. Open circles (o) show larvae undergoing notochord flexion.

or Ovaprim also resulted in hormonally assisted natural spawning of viable eggs, females treated with hCG did not spawn naturally and eggs had to be stripped manually. In addition, eggs released by females treated with either LHRH-a or Ovaprim were fertilized by the males in the tanks.

Description of eggs

Eggs are spherical and positively buoyant, have a smooth, unpigmented chorion, and range between 960 and 1200 μm in diameter (1039 ± 5 μm, mean ± 2 s.e.; *n* = 192). No significant differences were found in the diameters of naturally and hormonally induced spawned, fertilized and unfertilized eggs (ANOVA, *P* > 0.05; Table 2). Late-stage fertilized eggs possess a large pigmented oil globule (240 ± 6 μm diameter, mean ± 2 s.e.; *n* = 24) located anteriorly in the unsegmented yolk. Eggs hatched in ~32 h at ~23°C.

Identification of larvae

Identification of the reared larvae as those of *G. hebraicum* Richardson, 1845 was confirmed by comparing the fin meristics obtained from the largest larva in our series with that provided for adults of this species in the literature (D VIII, 11; A III, 9; P$_1$, 16; P$_2$ I, 5; McKay 1997). A developmental series from the largest to the smallest larva was assembled using body shape, head spines, gut shape, sequence of fin development, and pattern of pigmentation.

Description of larvae

Age and size range of developmental stages. Newly hatched yolk-sac larvae (0 h) measured between 1.75 and 2.10 mm (Figs 1, 2). The yolk sac was completely resorbed after 3.3 days. The largest yolk-sac larva measured 2.60 mm and the smallest larva without a yolk sac measured 2.30 mm (Table 2). The preflexion stage, i.e. after yolk-sac resorption and before the flexion stage, lasted about 15 days, with larvae ranging between 2.30 and 3.95 mm (Fig. 1). Flexion of the notochord commenced around Day 15 and was complete between Days 25 and 30. The smallest and largest larvae undergoing flexion measured 3.42 (Day 15) and 4.80 mm

(Day 27), respectively. The smallest postflexion larva measured 4.83 mm and corresponded in morphology to a 25-day-old larva (Table 3; Fig. 1).

Morphology. Larvae are very elongate to moderate in depth during the yolk-sac to preflexion stage (BD 7.8–27.3%), gradually becoming deep by the postflexion stage as the gut enlarges and becomes voluminous (BD 36.8–50.7%; Table 3). The snout is round and short, and the head becomes large from the flexion stage (HL 34.9–50.0%), reaching to ~60% of the body length in postflexion larvae (mean 43.1%). Both the relative depth of the body and the head length remain similar and increase at a similar rate during development. The relative distance between the snout and the anus remains long throughout development (PAL 54.1–68.3%, Table 3), as the gut does not lengthen with growth.

Newly hatched larvae possess a moderately large, unsegmented yolk sac (length across 48.8–63.8% BL) with a large, pigmented oil globule located anteriorly in the sac, and they have no formed eyes, mouth or pectoral-fin buds (Fig. 2A). The eyes are barely distinguishable and unpigmented in yolk-sac larvae but are developed, round and fully pigmented in early preflexion larvae from 2.30 mm (3 days; Fig. 2B). The mouth becomes functional in preflexion larvae from Day 3 and first feeding (rotifers) occurs after 3.5 days. Small villiform teeth develop along the premaxilla in preflexion larvae from 2.60 mm (7 days) and along the dentary from 3.12 mm (11 days; Fig. 2C). The gill membranes are free from the isthmus. A small inflated gas bladder is evident from the late preflexion stage from 2.45 mm (4.3 days). The gut in newly hatched larvae has a distinctly coiled foregut and an uncoiled, elongate hindgut; the gut becomes voluminous in late preflexion larvae, and triangular and compact in late postflexion larvae (Figs 2B–2H). A short preanal membrane is present until the late flexion stage (Figs 2B–2F), and there is no gap between the anus and the origin of anal fin. There are 24–25 myomeres (10 + 15 = 25 vertebrae; McKay 1997). Scales start to form in late postflexion larvae from about 7.00 mm (37 days) and were still developing in the largest larva examined (8.13 mm; 45 days) (Fig. 2H).

Larvae possess small to moderate head spination comprising anterior and posterior preopercular spines, opercular, subopercular, interopercular, posttemporal and supracleithral spines, a small sphenotic ridge and a smooth supraocular ridge (Figs 2D–2H). Two posterior preopercular and 3–5 anterior preopercular spines appear during the late preflexion and flexion stage, respectively. Posterior and anterior preopercular spines increase to 7–10 and 8–10 spines from 6.02 mm (37 days), respectively, and remain small during development; both sets of spines are present in the largest larva examined (Fig. 2H). A small subopercular

spine forms by 4.20 mm (19 days), and one small interopercular spine by 4.80 mm (19 days); the subopercular spine remains single while the number of interopercular spines increases to 4–5 by 6.02 mm (37 days). An opercular spine develops from 4.60 mm (19 days). The smooth supraocular ridge forms during the flexion stage (Figs 2E, 2F) and becomes prominent from 5.16 mm (30 days). The small sphenotic ridge develops from 5.16 mm (30 days) and becomes blunt from 7.00 mm (45–46 days). A low posttemporal ridge forms during the flexion stage and is present in the largest larva examined with up to four peaks (Fig. 2H). The supracleithral spine begins to form soon after notochord flexion is complete and develops 2–4 small peaks from 5.16 mm (30 days). All spines, and the supraocular and sphenotic ridges, were present in the largest larva and two juveniles examined (22.54–24.25 mm).

Development of fins. Pectoral-fin buds appear in early preflexion larvae from 2.30 mm (3 days), when the yolk sac is almost completely resorbed. Incipient pectoral-fin rays appear in late flexion larvae from 4.60 mm (19–20 days) and all rays (16) are formed in postflexion larvae from 6.10 mm (37 days; Figs 2E, 2F, 2H). Pelvic-fin buds, and dorsal-, caudal- and anal-fin anlagen appear simultaneously in late preflexion larvae from 3.82 mm (15 days; Fig. 2D). Incipient pelvic-fin rays are present in flexion larvae from 4.00 mm (19 days) and all elements (I, 5) are developed in early postflexion larvae from 5.16 mm (30 days). Incipient dorsal- and anal-fin rays appear in flexion larvae from 4.00 mm (19 days), and all elements are present in early postflexion larvae from 5.16 mm (30 days) except for the posterior-most spines of both the dorsal (VIII) and anal (III) fins which at that size are still soft rays (Fig. 2G). The full complement of dorsal-fin spines and rays (VIII, 11) is present in late postflexion larvae from 6.60 mm (45 days), and the third anal-fin spine is visible in the largest larva examined (8.13 mm, 45 days; Fig. 2H). Caudal-fin rays begin to form during notochord flexion, and the full complement (9+8) is evident in postflexion larvae from 5.16 mm (30 days). On the basis of the timing of development of all elements in each fin, the sequence of fin formation can be expressed as P_2, $C \rightarrow P_1 \rightarrow D \rightarrow A$.

Pigmentation. Larvae are initially moderately pigmented, becoming heavily pigmented with growth. Pigment intensity, as well as types of melanophores present (e.g. punctate and/or stellate), varied considerably among the larvae examined (Figs 2A–2H). Newly hatched yolk-sac larvae possess pigment on the anteriorly located oil globule, diffuse melanophores scattered dorsally along the body, a melanophore on the foregut and the hindgut above the anus, and one large melanophore midway ventrally on the tail (Fig. 2A). In live, newly hatched yolk-sac larvae (not

illustrated), two of the dorsal melanophores, i.e. the one above the foregut and the one midway along the tail, are prominent and spread into the dorsal finfold, whereas the large ventral tail melanophore spreads into the ventral finfold.

Head pigment is restricted to one melanophore on the lower jaw in preflexion larvae, increasing to up to five distinct melanophores along the ventral midline of the lower jaw in flexion larvae (Figs 2B–2F). Pigment on the head intensifies with growth and becomes heavy in late postflexion larvae, with melanophores densely distributed all over the head including the upper and lower jaw tips and lower jaw angle, along the premaxilla and dentary, along the isthmus and at the cleithral symphysis, and on the subocular and opercular areas (Figs 2G, 2H). The characteristic diagonal dark band across the eye was forming in the two juveniles examined (22.54–24.25 mm) and is present in juveniles >30 mm (>100 days) (Fig. 3). Internal pigment on the head is visible until the early postflexion stage and consists of a few melanophores around the upper jaw tip, the forebrain, ventrally along and on the junction of the mid and hindbrain, and along the cleithrum (Figs 2C–2G).

Dorsal body pigment in preflexion larvae consists of three prominent melanophores — on the nape, midway along the trunk and on the anterior portion of the tail — all of which remain prominent until the late preflexion stage (Figs 2B–2D); the nape melanophore becomes internal by the flexion stage. Ventral body pigment in preflexion larvae consists of a single melanophore on the gut, one at the anus, and up to four ventrally along the tail. Pigment dorsally on the trunk and tail, and ventrally on the gut and tail, intensifies with growth and expands laterally over the body including the trunk myosepta, covering all except the last 6–7 caudal myomeres in late preflexion to late flexion larvae, and the last 3 caudal myomeres in postflexion and late postflexion larvae (Figs 2E–2H). The entire caudal peduncle was pigmented in the juveniles examined. The gut becomes heavily pigmented in late postflexion larvae (Fig. 2H). Internal pigment is present over the gas bladder in preflexion larvae from 2.45 mm, and dorsally along the gut and notochord, and remains visible until the late flexion stage (Figs 2E, 2F) before becoming obscured by the external pigmentation. The characteristic six horizontal dark bands along the body, which usually disappear in older adults, were present in the juveniles examined (Fig. 3).

Pigment on the pelvic-fin buds starts to form during the late flexion stage and the fins become heavily pigmented from the early postflexion stage (Fig. 2G); both fins were also heavily pigmented in the juveniles examined. All other fins remain unpigmented during development except for pigment on dorsal-fin spines I-VII and the interconnecting fin membrane, and the bases of the second dorsal and anal fin (Fig. 2H). The pectoral-fin base becomes pigmented

during the late postflexion stage. No pigment was present on the second dorsal, caudal, anal and pectoral fins in the juveniles examined.

Discussion

Hormone-induced spawning

Preliminary results of this study showed that hormonal maturation and ovulation of WA dhufish, *Glaucosoma hebraicum*, as well as the artificial rearing of eggs through to the juvenile stage, could be successfully achieved. The minimum total lengths of sexually mature dhufish found in this study, i.e. 47 cm for males (m) and 43 cm for females (f), were greater than those described by Hesp (1997) (m, 35–40 cm; f, 25–30 cm). Whereas most male dhufish matured naturally, wild-caught females did not readily spawn in captivity but responded well to hCG, LHRH-a and Ovaprim. Both hCG and LHRH-a have been successfully used to induce oocyte maturation in several other marine finfish species, including hCG in grouper (*Epinephelus striatus*) and snapper (*Pagrus auratus*), and LHRH-a in milkfish (*Chanos chanos*) and barramundi (*Lates calcarifer*) (Lee et al. 1986; Garcia 1989; Battaglene and Talbot 1992; Watanabe et al. 1995). In addition, Ovaprim has been used to induce ovulation in several freshwater fish species (Thomas and Boyd 1989). The dosage of LHRH-a *imp* that provided best results for inducing maturation in dhufish lies within the range employed in other marine fishes (Garcia 1989; Mayes et al. 1993).

Although females injected with low dosages of either LHRH-a or Ovaprim ($\leq$13 μg kg^{-1}) spawned unaided, those treated with high dosages ovulated but did not spawn naturally, and eggs had to be stripped manually. Manual stripping of eggs resulted in spawns being wasted because of difficulty in assessing the exact ovulation time, poor fertilization rates, and high (30%) loss of female broodstock through repeated handling. A delay of even a few hours in stripping mature eggs can affect fertilization success in some temperate fishes (Shelton 1989), and finfish species that are susceptible to handling stress during the spawning period may fail to ovulate or may ultimately die as a result of the treatment (Billard et al. 1981). Although hormonal induction leading to unaided spawning is relatively uncommon (e.g. Kuo et al. 1988), it can result in single ovulations per season and poor fertilization rates in species such as snapper and milkfish (Lee and Tamaru 1987; Battaglene and Talbot 1992; Pankhurst and Carragher 1992).

Larval development and relationships

This paper presents the first account of the early life-history stages of the percoid family Glaucosomatidae. As with most cases in which the description of larval stages of a fish species is based solely on artificially reared material,
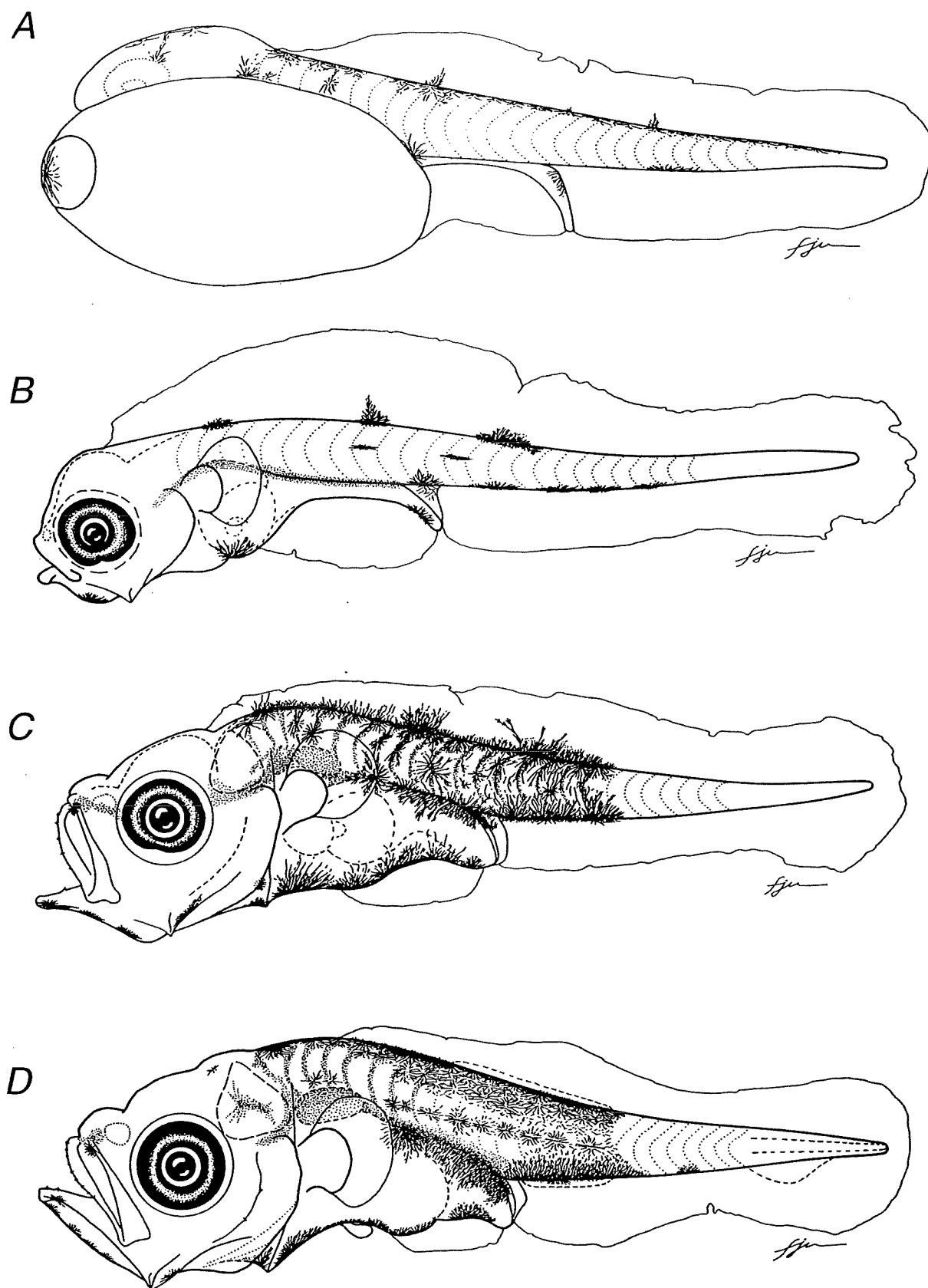
Fig. 2. Larvae of *Glaucosoma hebraicum* reared in laboratory. *(A)* 2.05 mm BL newly hatched; yolk sac (0 h). *(B)* 2.43 mm BL preflexion larva (3.3 days). *(C)* 3.16 mm BL preflexion larva (11 days). *(D)* 3.82 mm BL late preflexion larva (15 days). *(E)* 4.62 mm BL flexion larva (19 days). *(F)* 4.65 mm BL flexion larva (19 days); note expanded melanophores compared with larva illustrated in E. *(G)* 5.35 mm BL early postflexion larva (35 days); only last five caudal myomeres drawn. *(H)* 8.13 mm BL late postflexion larva (45 days); developing scales and last caudal myomeres not drawn. Illustrated by F. J. Neira.

E

F

G

H

Fig. 2. *continued*

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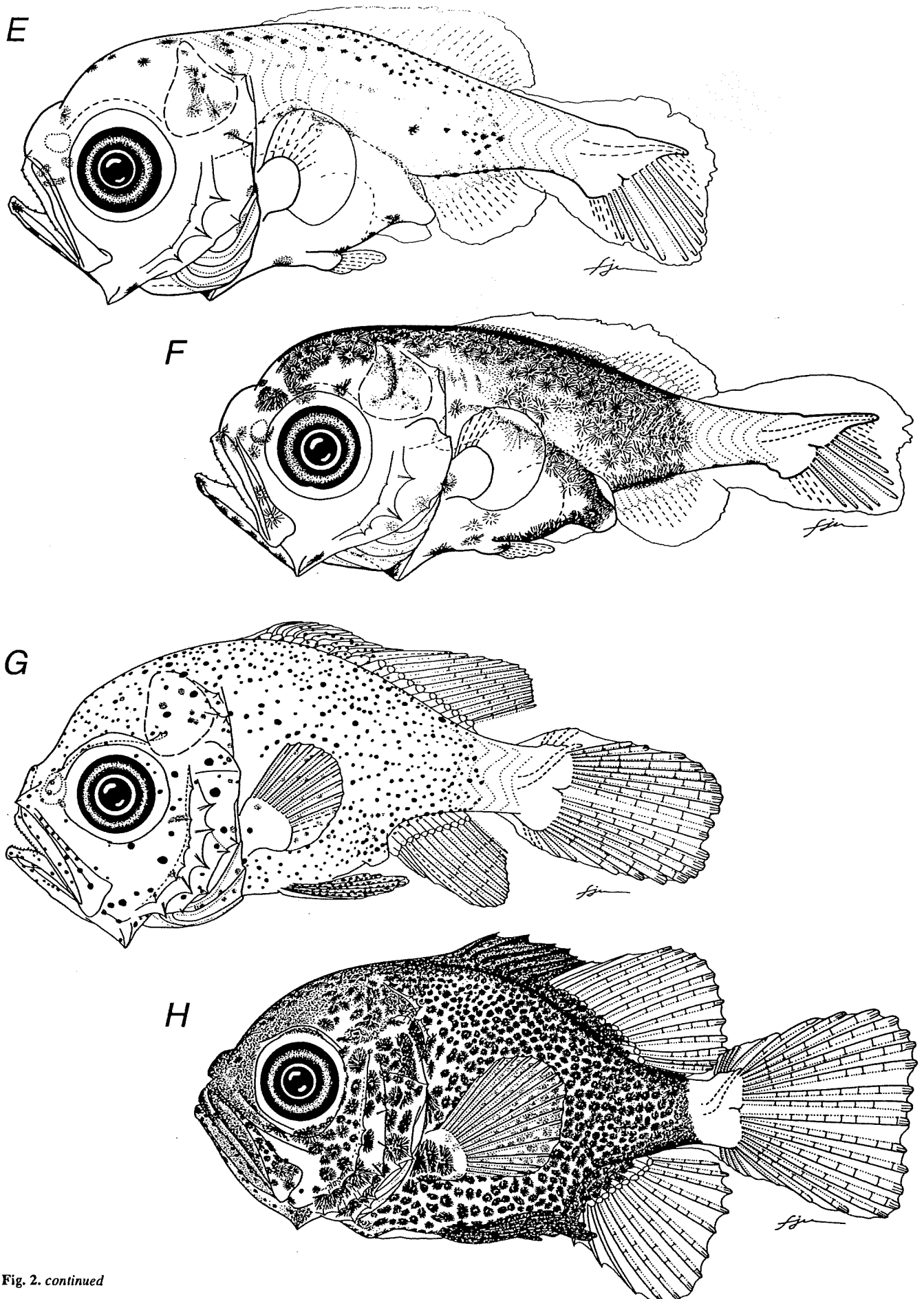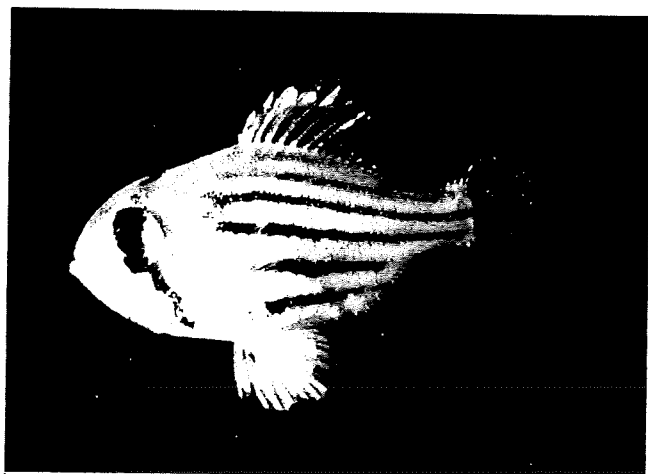
Fig. 3. 31.0 mm BL reared juvenile of *Glaucosoma hebraicum* (105 days) (photographed by J.B.Hutchins, Western Australian Museum, Perth).

it should be borne in mind that *G. hebraicum* larvae described herein may differ to some extent from field-caught larvae, given the range of conditions in rearing tanks (e.g. light intensity, temperature, larval density, food supply) and fixation time (e.g. before or after death). For example, the marked difference in pigment intensity between the two 19-day flexion *G. hebraicum* larvae drawn in our series (cf. Fig. 2*E*, 2*F*) illustrates the variability that can occur even at the same developmental stage with reared material. However, the difference between these flexion larvae (and also among several other larvae examined) seems to be associated with the expanded nature of the melanophores and not, as is often the case with reared larvae (Hunter 1984), with the presence of more pigment cells in the heavily pigmented specimen. In addition to pigment intensity, reared larvae may also differ from wild-caught larvae in those morphological characteristics that are partially controlled by environmental conditions during rearing, such as fin-ray counts (Hunter 1984). However, counts of all fin spines and rays in our reared material were identical to those provided for adult *G. hebraicum* in the literature (McKay 1997).

Reared larvae can be assigned ages (hours, days) to describe developmental stages and the duration of developmental events. During this study, both the preflexion (after yolk-sac resorption) and flexion stages lasted for several days: 3–15 and 16–27 days, respectively. In contrast, the size range of these stages was markedly narrow: 2.30–3.95 and 3.42–4.80 mm, respectively (Table 3; Fig. 1). The small variation in length compared with duration (days) of these stages implies that it could be highly misleading to assign ages to field-caught larvae based on reared material. In addition, 37–40-day-old postflexion larvae ranged between 6.02 and 7.48 mm, whereas 45–46-day-old late postflexion larvae ranged between 6.35 and 8.13 mm,

suggesting that size increments of larvae near transition to the juvenile stage decreased with increasing days after birth. A decrease in larval growth near the juvenile stage has also been described for other artificially reared marine fishes, including red sea bream (*Pagrus major*) and starry flounder (*Platichthys stellatus*) (Policansky 1982; Fukuhara 1991). As with sea bream, size rather than age of larval *G. hebraicum* is probably more suitable for estimating the onset of transition to the juvenile stage. Although the precise size range at which this transition takes place in *G. hebraicum* could not be determined from our reared series, it possibly starts at around 8 mm (45–46 days) because the largest postflexion larva examined (8.13 mm) had attained the full complement of external meristic characters, except for the scales which were still developing.

Except for the early-forming pelvic fins, the development of larval *G. hebraicum* follows a pattern similar to that found in most generalized percoids (Johnson 1984). This includes the formation of the caudal, dorsal and anal fin anlagen shortly before the start of the notochord flexion, a size at flexion between 3 and 5 mm which ends with the attainment of the full complement of caudal-fin rays, and the presence of spines on bones of both the opercular (preopercle, opercle, subopercle and interopercle) and pectoral (supracleithrum and posttemporal) series (Johnson 1984). In addition, the last dorsal-fin spine (VIII) and the last anal-fin spine (III) are initially soft rays but become spines during the postflexion stage, as with the larvae of most percoids (Leis and Trnski 1989; Neira *et al.* 1998). The precocious development of the pelvic fins constitutes the only apparent specialization of *Glaucosoma* larvae to pelagic life. This pattern of early-forming pelvic fins has also been observed in larvae of a few other percoid families, including the Lutjanidae, Monodactylidae (*Monodactylus* only), Pempheridae and Serranidae (Leis and Rennis 1983; Miskiewicz 1989), and in larvae of some members of non-percoid taxa such as beryciforms and stromateoids (Horn 1984; Neira *et al.* 1998).

Glaucosoma, the sole genus of the Glaucosomatidae, shares several anatomical similarities with members of the percoid family Pempheridae, including the structure of the gas bladder, the foramen on the frontal bone and the lack of direct articulation between the second epibranchial and the pharyngobranchial (Katayama 1954; Tominaga 1986; Johnson 1993). These, including the similarities between the saccular otoliths (sagittae) of *G. magnificum* and those of *Pempheris* (McKay 1997), seem to further support the close relationship *Glaucosoma*-Pempheridae that led Johnson (1993) to recommend the inclusion of the Glaucosomatidae as a subfamily of the Pempheridae. *Glaucosoma* larvae described here share several similarities with pempherid larvae, including the moderately deep body, myomere number (24–25), the early-forming pelvic fins, a similar size range at notochord flexion (3.6–4.3 mm in pempherids), the pigment pattern on the body and gut in the early stages, the

compact triangular gut, and the lack of a gap between the anus and the anal-fin origin. However, they also differ in several respects: the pelvic fins in *Glaucosoma* are initially unpigmented and form ventrally on the gut during the late preflexion stage, whereas in pempherids they are pigmented and appear first laterally on the gut in the yolk-sac stage and then migrate ventrally and anteriorly; the foregut is coiled in early preflexion *Glaucosoma* larvae, but it only starts to coil by the mid-preflexion stage in pempherid larvae; *Glaucosoma* larvae possess a more extensive head spination that includes more spines in the preopercular series, and also suborpercular, interopercular and posttemporal spines, and supraocular and sphenotic ridges, all of which are absent in pempherid larvae; and *Glaucosoma* larvae lack the prominent dorsal finfold of pempherid larvae, and are generally much more heavily pigmented than pempherid larvae (Leis and Rennis 1983; Kinoshita 1988). Since there is no evidence to suggest that the similarities between *Glaucosoma* and pempherid larvae are derived, no conclusions can be drawn as to whether these support the view that both groups are closely related; this in turn illustrates the difficulty in assessing relationships among percoids by using larval characters alone (e.g. Neira *et al.* 1997).

Taxa with similar larvae

Larval *G. hebraicum* can be identified by the body shape, the presence of 24–25 myomeres, the early-forming abdominal pelvic fins that become heavily pigmented by the early postflexion stage, the gut that is compact and triangular-shaped from the flexion stage, the head spines, and the distinct body pigment from the late flexion stage. Field-caught *G. hebraicum* larvae are likely to be confused with those of the two other species of *Glaucosoma* found in Western Australia, namely *G. buergeri* and *G. magnificum*. Larvae of those species are undescribed but would be unlikely to co-occur with those of *G. hebraicum* since both are tropical species that are found mostly north of Shark Bay along northern Australia, including off Papua New Guinea. In addition, while dorsal and anal fin counts in *G. hebraicum* are identical to those of *G. buergeri* (D VIII,11; A III, 9), gill rakers could be used to separate late postflexion larvae of both species (4–6 + 11–13 *v.* 6–9 + 13–15), whereas dorsal and anal fin counts will separate postflexion larvae of both species from those of *G. magnificum* (D VIII,14; A III,12) (Allen and Swainston 1988; Paxton and Hanley 1989; McKay 1997).

Glaucosoma larvae can also be confused with larvae of members of the Pempheridae and with *Monodactylus* (Monodactylidae), which are found within the distributional range of *Glaucosoma* (Allen and Swainston 1988) and which possess similar body shapes and early-forming pelvic fins. However, in addition to the distinguishing features between *Glaucosoma* and pempherid larvae mentioned above, larvae of both groups can be further separated by the

higher number of anal-fin rays in pempherids (17–45 *v.* 9–12), whereas *Monodactylus* larvae possess a distinct wide band of heavy pigment between the snout and the anus, and a higher number of dorsal and anal-fin rays (27–32) (Leis and Rennis 1983; Kinoshita 1988; Miskiewicz 1989). Larvae of *Glaucosoma* could also be confused with larvae of non-percoids that have early-forming pelvic fins, including members of the Berycidae and Trachichthyidae (Beryciformes), and some stromateoids. However, berycids are lightly pigmented and have I, 7–13 pelvic-fin elements (Mundy 1990; Miskiewicz *et al.* 1998), trachichthyids possess 26–30 myomeres and have I, 6 pelvic-fin elements (Jordan and Bruce 1993), and stromateoids usually have 30 or more myomeres (Horn 1984).

Acknowledgments

References

Allen, G. R., and Swainston, R. (1988). 'The Marine Fishes of North-Western Australia: a Field Guide for Anglers and Divers.' (Western Australian Museum, Perth.) 201 pp.

Anon. (1997). Australian Fisheries Statistics. (Australian Bureau of Agricultural and Resource Economics: Canberra.)

Battaglene, S. C., and Talbot, R. B. (1992). Induced spawning and larval rearing of snapper, *Pagrus auratus* (Pisces : Sparidae), from Australian waters. *New Zealand Journal of Marine and Freshwater Research* 26, 179–83.

Battaglene, S. C., and Talbot, R. B. (1994). Hormone induction and larval rearing of mulloway, *Argyrosomus hololepidotus* (Pisces : Sciaenidae). *Aquaculture* 126, 73–81.

Billard, R., Bry, C., and Gillet, C. (1981). Stress, environment and reproduction in teleost fish. In 'Stress and Fish'. (Ed. A. D. Pickering.) pp.185–208. (Academic Press: London.)

Cockman, B. W., and McGuire, B. (1986). Age, growth and spawning of the Westralian dhufish *Glaucosoma hebraicum* Richardson (Pisces : Glaucosomidae). Western Fisheries Research Committee – Annual Reports and Proposals, pp. 205–7. (Fisheries Department of Western Australia: Perth.)

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Fukuhara, O. (1991). Size and age at transformation in red sea bream, *Pagrus major*, reared in the laboratory. *Aquaculture* 95, 117–24.

Garcia, L. M. (1989). Dose-dependent spawning response of mature female sea bass, *Lates calcarifer* (Bloch), to pelleted luteinizing hormone-releasing hormone analogue (LHRHa). *Aquaculture* 77, 85–96.

Hesp, S. A. (1997). The biology of the dhufish, *Glaucosoma hebraicum*, in offshore waters on the lower west coast of Australia. BSc (Hons) Thesis, Murdoch University, Perth. 60 pp.

Horn, M. H. (1984). Stromateoidei: development and relationships. In 'Ontogeny and Systematics of Fishes'. (Eds H. G. Moser, W. J. Richards, D. M. Cohen, M. P. Fahay, A. W. Kendall Jr and S. L. Richardson.) pp. 620–8. American Society of Ichthyologists and Herpetologists Special Publication No. 1.

Hunter, J. R. (1984). Synopsis of culture methods for marine fish larvae. In 'Ontogeny and Systematics of Fishes'. (Eds H. G. Moser, W. J. Richards, D. M. Cohen, M. P. Fahay, A. W. Kendall Jr and S. L. Richardson.) pp. 24–7. American Society of Ichthyologists and Herpetologists Special Publication No. 1.

Hutchins B., and Swainston, R. (1986). 'Sea Fishes of Southern Australia: Complete Field Guide for Anglers and Divers.' (Swainston Publishing: Perth, Australia.) 180 pp.

Jenkins, G. I., and Frankish, K. (1997). The Fremantle Maritime Centre – active in supporting industry development. Aquaculture Council of Western Australia News 18, 24–8.

Johnson, G. D. (1984). Percoidei: development and relationships. In 'Ontogeny and Systematics of Fishes'. (Eds H. G. Moser, W. J. Richards, D. M. Cohen, M. P. Fahay, A. W. Kendall Jr and S. L. Richardson.) pp. 24–7. American Society of Ichthyologists and Herpetologists Special Publication No. 1.

Johnson, G. D. (1993). Percomorph phylogeny: progress and problems. *Bulletin of Marine Science* 52, 3–28.

Jonker, L. J. (1993). Comparisons of the larval fish assemblages in healthy and degraded seagrass meadows in Cockburn Sound, Western Australia. BSc (Hons) Thesis, Murdoch University, Perth. 47 pp.

Jordan, A. R., and Bruce, B. D. (1993). Larval development of three roughy species complexes (Pisces : Trachichthyidae), from southern Australian waters, with comments on the occurrence of *Hoplostethus atlanticus*. *Fishery Bulletin (US)* 91(1), 76–86.

Kailola, P. J., Williams, M. J., Stewart, P. C., Reichelt, R. E., McNee, A., and Grieve, C. (1993). 'Australian Fisheries Resources.' (Bureau of Resource Sciences: Canberra.) 422 pp.

Katayama, M. (1954). Systematic position of the genus *Glaucosoma*. *Bulletin of the Faculty of Education of Yamaguchi University* 4(1), 23–9.

Kendrick, A. J. (1993). Assemblages of larval fishes in coastal waters of south-western Australia. BSc (Hons) Thesis, Murdoch University, Perth. 88 pp.

Kinoshita, I. (1988). Pempheridae. In ['Atlas of the Early Stage Fishes in Japan']. (Ed. M. Okiyama.) pp. 507–9. (Tokai University Press: Tokyo.) (In Japanese.)

Kuo, C-M., Ting, Y-Y., and Yeh, S-L. (1988). Induced sex reversal and spawning of blue-spotted grouper, *Epinephelus fario*. *Aquaculture* 74, 113–26.

Lee, C-S., and Tamaru C. S. (1987). Application of LHRH-a cholesterol pellets to maturation of finfish: milkfish. *NOAA Technical Report* 85, 57–64.

Lee, C-S., Tamaru, C. S., Banno, J. E., and Kelley, C. D. (1986). Influence of chronic administration of LHRH-analogue and/or 17α-methyltestosterone on maturation in milkfish. *Aquaculture* 59, 147–59.

Leis, J. M., and Rennis, D. S. (1983). 'The Larvae of Indo-Pacific Coral Reef Fishes.' (New South Wales University Press: Sydney. University of Hawaii Press: Honolulu.) 269 pp.

Leis, J. M., and Trnski, T. (1989). 'The Larvae of Indo-Pacific Shorefishes.' (New South Wales University Press: Sydney.) 371 pp.

Mayes, K. B., Rosenblum, P. M., and Brandt, T. M. (1993). Raceway spawning of Florida largemouth bass: effects of acclimation time and hormone treatment on spawning success. *The Progressive Fish Culturist* 55, 1–8.

McKay, R. J. (1997). Pearl perches of the world (Family Glaucosomatidae). FAO Species Catalogue Vol. 17. FAO Fisheries Synopsis 125/17. 24 pp.

Miskiewicz, A. G. (1989). Monodactylidae: Diamonfishes, moonies, fingerfishes. In 'The Larvae of Indo–Pacific Shorefishes'. (Eds J. M. Leis and T. Trnski.) pp. 196–9. (New South Wales University Press: Sydney.)

Miskiewicz, A. G., Bruce, B. D., and Trnski, T. (1998). Berycidae: redfishes, nannygais, alfonsinos. In 'Larvae of Temperate Australian Fishes. Laboratory Guide for Larval Fish Identification'. (Eds F. J. Neira, A. G. Miskiewicz and T. Trnski.) pp. 95–6. (University of Western Australia Press: Perth.)

Mundy, B. C. (1990). Development of larvae and juveniles of the alfonsins, *Beryx splendens* and *B. decadactylus* (Berycidae, Beryciformes). *Bulletin of Marine Science* 46, 257–73.

Neira, F. J., Miskiewicz, A. G., and Bruce, B. D. (1997). Larvae of five fish families with pattern 10 of the *ramus lateralis accessorius* nerve (Arripidae, Girellidae, Kyphosidae, Microcanthidae and Scorpididae): relevance to relationships. *Bulletin of Marine Science* 60(1), 117–38.

Neira, F. J., Miskiewicz, A. G., and Trnski, T. (1998). 'Larvae of Temperate Australian Fishes. Laboratory Guide for Larval Fish Identification.' University of Western Australia Press, Perth. 475 pp. (In press.)

Pankhurst, N. W., and Carragher, J. F. (1992). Oocyte maturation and changes in plasma steroid levels in snapper *Pagrus* (=*Chrysophrys*) *auratus* (Sparidae) following treatment with human chorionic gonadotropin. *Aquaculture* 101, 337–47.

Paxton, J. R., and Hanley, J. E. (1989). Glaucosomatidae. In 'Zoological Catalogue of Australia, Vol. 7, Pisces: Petromyzontidae to Carangidae'. (Eds J. R. Paxton, D. F. Hoese, G. R. Allen and J. E. Hanley.) pp. 529–30. (Australian Government Publishing Service: Canberra.)

Policansky, D. (1982). Influence of age, size and temperature on metamorphosis in the starry flounder, *Platichthys stellatus*. *Canadian Journal of Fisheries and Aquatic Science* 39, 514–17.

Rowland, S. J. (1983). The hormone-induced ovulation and spawning of the Australian freshwater fish golden perch, *Macquaria ambigua* (Richardson) (Percichthyidae). *Aquaculture* 35, 221–38.

Rowland, S. J. (1988). Hormone-induced ovulation and spawning of the Australian freshwater fish Murray cod, *Maccullochella peelii* (Mitchell) (Percichthyidae). *Aquaculture* 70, 371–89.

Ruwald, F. P., Searle, L. D., and Oates, L. A. (1991). A preliminary investigation into the spawning and larval rearing of striped trumpeter, *Latris lineata*. Department of Primary Industry, Tasmanian Division of Sea Fisheries Technical Report No. 44. 17 pp.

Shelton, W. L. (1989). Management of finfish reproduction for aquaculture. *Aquatic Sciences* 1(3), 497–535.

Sudmeyer, J. E., Hancock, D. A., and Lenanton, R. C. J. (1990). Biological synopsis of Westralian jewfish (*Glaucosoma hebraicum*, Richardson, 1845) (Pisces : Glaucosomatidae). Western Australian Department of Fisheries, Fisheries Research Report No. 96. 20 pp.

Thomas, P., and Boyd, N. W. (1989). Dietary administration of an LHRH analogue induces spawning of spotted seatrout (*Cynoscion nebulosus*). *Aquaculture* 80, 363–70.

Tominaga, Y. (1986). The relationships of the families Glaucosomatidae and Pempherididae. In 'Indo–Pacific Fish Biology, Proceedings of the Second International Conference on Indo–Pacific Fishes'. pp. 595–9. (Eds T. Uyeno, R. Arai, T. Taniuchi and K. Matsuura.) (Ichthyological Society of Japan: Tokyo.)

Watanabe, W. O., Ellis, S. C., Ellis, E. P., Head, W. D., Kelley, C. D., Moriwake, A., Lee, C-S., and Bienfang, P. K. (1995). Progress in controlled breeding of Nassau grouper (*Epinephelus striatus*) broodstock by hormone induction. *Aquaculture* 138, 205–19.

Appendix N   Developing a weaning technique for dhufish larvae

Introduction

Although larval rearing of WA dhufish has been moderately successful (up to 37% survival to metamorphosis; Payne *et al.*, 2000), weaning has proved to be difficult. Due to the highly selective feeding nature of juvenile dhufish and the limited number of fish available for trials, preliminary attempts to wean the larvae onto an artificial diet were unsuccessful. It was observed that ingestion of the commercially available weaning diet was low, resulting in high mortality during weaning. As pellet attractiveness is one of the key requirements for a successful weaning diet, in the current study a range of techniques for improving pellet attractiveness were compared in 2 separate studies.

The first experiment compared a commercial diet (Nippai ML) with a microbound diet (MBD; prepared by blending fresh fish flesh, enriched artemia meta-nauplii and the commercial diet) and live-feed diet (newly hatched snapper larvae, *Pagrus auratus*). A second experiment compared preparation techniques of the fresh MBD (fridge-dried vs oven-dried MBD) and also investigated a simple way of potentially improving the attractiveness of the commercially available diet by coating it with a fish emulsion. These were compared to the commercial diet (control). The performance of each diet was assessed in terms of growth, survival and observations of the interest of the fish in the pellet (ie number of strikes at pellet per 2 minutes).

Methods

Source of larvae

Experiment 1

Larvae were obtained from stripping eggs and sperm from hormone treated captive dhufish. Larvae were reared in a flow-through system in a series of 140L cylindrical tanks with blue walls and white bottoms at an initial stocking density of 6 larvae.L^{-1}. Rearing is described in Payne *et al.* (2000). Larvae were fed on cultured copepod nauplii reared on *T-Iso* and rotifers enriched with Super Selco®. At 6mm in length, larvae were weaned onto artemia metanauplii enriched on Super Selco® and were then fed 4 times daily to satiety, and also readily consumed broodstock calanoid copepods resident in the tank.

Experiment 2

Eggs were obtained as for experiment 1. Larvae were reared in a semi-intensive system modified from Partridge *et al.*. (1998) in a 5,000 litre cylindro-conical tank. Larvae fed on rotifers (self-maintaining population in tank feeding on microalgae) and natural blooms of harpacticoid

copepods. When larvae measured 5.7 mm, *Artemia* metanauplii enriched with Super Selco® were fed 4 times daily to satiety.

Experimental system

Each dietary treatment was investigated in triplicate in 160-L blue cylindrical tanks with white bases. Each tank was fitted with 800μm outlet screens and supplied with seawater (filtered to 1μm) at 1 litre.minute^{-1} and aeration. Temperature and dissolved oxygen were measured daily and adjustments made to maintain similar conditions between tanks. Each tank was vacuumed as required (up to 2 times daily). Larvae were stocked to the experimental system by slowly draining the larviculture tank and, using a beaker to gently scoop up the larvae and transfer them to the experimental tanks. Larvae continued to be fed solely on enriched Artemia nauplii until experimental Day 2 when the weaning diets were introduced.

Experiment 1

Larvae were harvested from the their tanks on Day 28 post-hatch. Ninety-nine fish (mean length 13.17 ± 0.36 mm, n = 10) were stocked into each of 9 x 160 litre cylindrical tanks (described above). Tanks were allocated randomly in triplicate to one of three treatments of different weaning diets: live feed, commercial pellet (ML) or fridge-dried micro bound diet (fridge-dried MBD).

Experiment 2

When larvae exceeded 12mm (Day 45 post-hatch), seventy-four dhufish larvae were stocked into each of twelve tanks described above. Tanks were randomly allocated in triplicate to one of four treatments of different weaning diets: commercial pellet, commercial pellet sprayed with fish emulsion (Spray ML), fridge-dried microbound diet (fridge-dried MBD) and oven-dried microbound diet (Oven-dried MBD).

Weaning strategy

The basic weaning strategy for experiments 1 and 2 was to reduce the number of artemia feeds per day from four (Days 1-9) to three (Days 10-30) and then to gradually reduce the amount of artemia per feed. The amount of artemia per feed was initially 30,000 (Days 1-11). Beginning on Day 12, this was gradually reduced to zero by Day 32. Concurrent to reducing the artemia feeding regime, the number of feeds of the alternate diet was increased from four (Days 3-10) to nine (Days 11-18). From Day 19, in Experiment 1 the alternate diet was presented 19 times per day, while in Experiment 2 for logistical reasons, it was presented 9 times only. For pelletised weaning diets, the size of the pellet was increased gradually: 200-400 □m (Days 3-13); 300-600 □m (14-19); 500-800 □m (Days 20-30); 700-1100 □m (Days 31-43). At each feed, the weaning

diets were offered *ad libitum* before the artemia were offered. For pelletised diets, the amount of diet fed was the same among treatments (by volume).

Diets

Live feed

Fertilised snapper eggs were collected from the overflow of a tank containing naturally spawning snapper. These were hatched and fed to the dhufish on either the day of hatch or Day 1 post-hatch. The number of larvae were estimated volumetrically. The number of snapper larvae per feed was increased throughout the experiment: 4 x 500 larvae (Day 3 - 10); 9 x 600 larvae (Day 11-12); 9 x 1000 (Day 13-14); and 9 x 1500 (Day 15-18); and 19 x 1500 (Day 19-43).

Commercial diet (ML)

Nippai ML was used with a size range increasing from 300μm on Day 3 to 1000μm on Day 31 according to the schedule above.

Micro-bound diets (fridge-dried and oven-dried)

Minced flesh (30% mussel meat, 20% mulies, 20% whiting, 20% prawns and 10% squid), artemia and Nippai ML were blended into a homogenous paste. Small volumes of water added as required to achieve a thick paste. The diet mixture was bound with gelatin at 7.5% by wet weight as follows. Gelatin was dissolved in hot water and then cooled to 40°C. It was then added to the diet mixture and thoroughly mixed. The diet was cast onto trays to a depth of approximately 300 μm. The trays were placed in the fridge at 14°C for 24h (fridge-dried MBD) or in oven at 50°C until dry (oven-dried MBD). Once the diet is dried it was chopped and sieved to the required size.

At the start of the wean the MBD diet (oven-dried and fridge-dried) contained a low proportion of ML (20%)and a high proportion of minced flesh (70%). During the course of the wean, the proportion of Nippai ML increased gradually to 100% while the concentration of flesh decreased to 0 % (according to Table A22). The proportion of artemia remained at 10% throughout the wean, until the proportion of Nippai reached 90%, when it reduced to 2% then 0%.

Spray-coated ML

This diet was prepared by spraying ML pellet with homogenised mulies prior to feeding. Spraying occurred each morning, prior to the first feed and the coated pellet was stored in an airtight container in the fridge for the remainder of the day. Spraying continued until Day 40 of the trial, after which straight ML was used.

Table 22: **Composition of microbound diet used in Experiment 1 and 2.**

Days of Trial	Nippai ML (%)	Minced flesh* (%)	Enriched *Artemia* (%)
3-13	20	70	10
14-17	30	60	10
16-20	40	50	10
19-25	50	40	10
24-26	60	30	10
27-29	70	20	10
27-32	80	10	10
32-37	90	5	2
35-40	100	0	0
39-43	ML only	0	0

* mussel meat 30%, oily fish fillets 20%, lean fish fillets 20% prawn meat 20%, squid meat 10%.

Data collection

Interest in weaning diet: Observations on the larvae's response to the alternate food source were made during the first feed on each day. During this feed, two observers observed each tank for 2 minutes immediately follow the addition of the artificial diet. A number of fish observed striking pellets were counted and recorded.

Growth: A random sample of 10 fish were weighed and measured (SL) on Day 0 (SL only), 20 and 45. The fish in each tank were lightly anaesthetised (40 ppm benzocaine) and 10 fish from each tank were caught individually in fine gauze dip-net, weighed, measured and recovered. The remainder of the fish in each tank were removed and placed into recovery bucket while the tank was refilled.

Survival: Mortalities in each tank were removed and recorded daily.

Statistics

Survival on Day 45 was compared by 1 way ANOVA for each experiment. Weight was compared by 2 way ANOVA for each experiment. Comparisons of means were conducted by Tukey Kramer HSD. $\alpha=0.05$.

Results

Experiment 1

Overall survival for fridge-dried MBD and live feed treatments were significantly greater than for the ML treatment (P<0.05; Fig A40a). Mortalities began on day 18 in both the pelletised treatments. Mortalities in the live-feed treatment first occurred slightly later at day 20. The amount of artemia being fed at this stage was less than 1000 nauplii per day, and the number of feeds of the alternate diet increased from 9 to 19. Mortalities had virtually ceased by Day 25.

Fish in all treatments grew significantly between day 20 and 35, with wet weight increasing from approximately 0.1g at day 20 to between 0.6 and 1 g by day 35 (P<0.01; Fig A41a). At day 20 there was no significant difference in wet weight between treatments, however, by day 35 fish in the fridge-dried and live-feed treatments were significantly heavier than the ML treatment (P<0.01). A similar pattern was found for SL (P<0.01; data not shown).
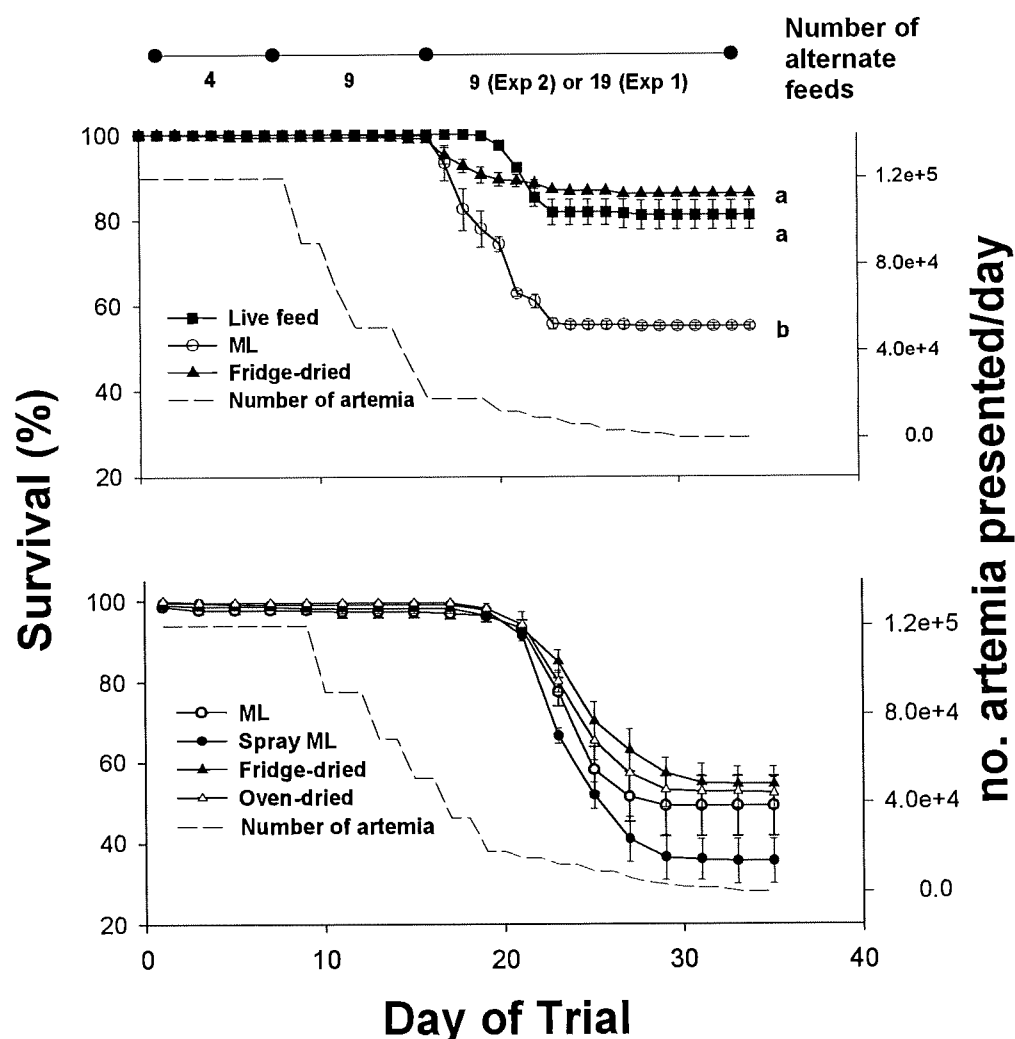
Figure A40: Survival of dhufish larvae during weaning experiments 1 and 2. Superscripts indicate significantly different Tukey-Kramer HSD groupings at Day 35 (P<0.05)

Strike rate was difficult to assess for the live-feed group because the snapper larvae were too small to be seen clearly. In addition the feeding response to the live feed was usually too vigorous to count with any degree of accuracy. Consequently the variation in counts was high and likely to be meaningless. Therefore this data is not presented here. Striking at the fridge-dried MBD began on day 14 and striking at the ML occurred a day later (Day 15; Fig A42a). The number of artemia being presented on day 14 and 15 was approximately 40,000 artemia per day. Following the first strike, strike rates rapidly increased in both treatments, however, striking on the fridge-dried MBD was higher than on ML.
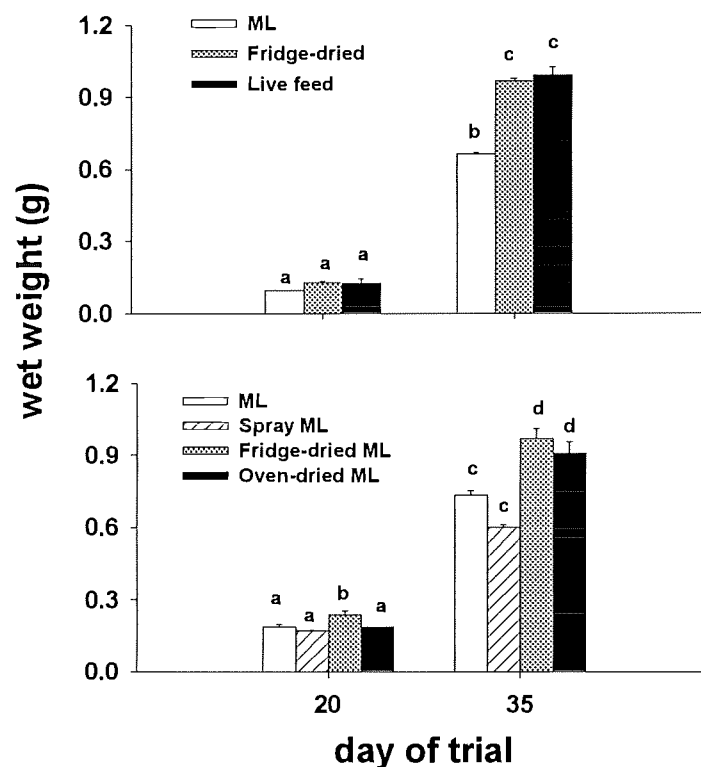
Figure A41: Wet weight of dhufish larvae at 20 and 35 days during weaning experiments 1 and 2. Superscripts indicate significantly different Tukey-Kramer HSD groupings (P<0.05)

Experiment 2

Although there was a small number of mortalities in all treatments prior to Day 18, the majority of mortalities were experienced after this time (Fig A40b). The concentration of artemia at this time was less than 10,000 per day. There was no significance in survival at day 35 between the four treatments (due to large standard errors; P>0.05), however, in the spray ML treatment survival was approximately 70% of the other three treatments. Mortalities had virtually ceased in all treatments by day 31.

Larvae in all treatments grew significantly between day 20 and 35 (P<0.05), with wet weight increasing significantly from approximately 0.15 at day 20 to 0.6 – 1 by day 35 (Fig A41b). At day 20 wet weight in fish in the fridge-dried MBD treatment was significantly greater than the other three groups. By day 35, wet weight of the ML and spray ML groups were similar to each other but significantly less than both the fridge- and oven-dried groups. A similar pattern was found for SL (data not shown).
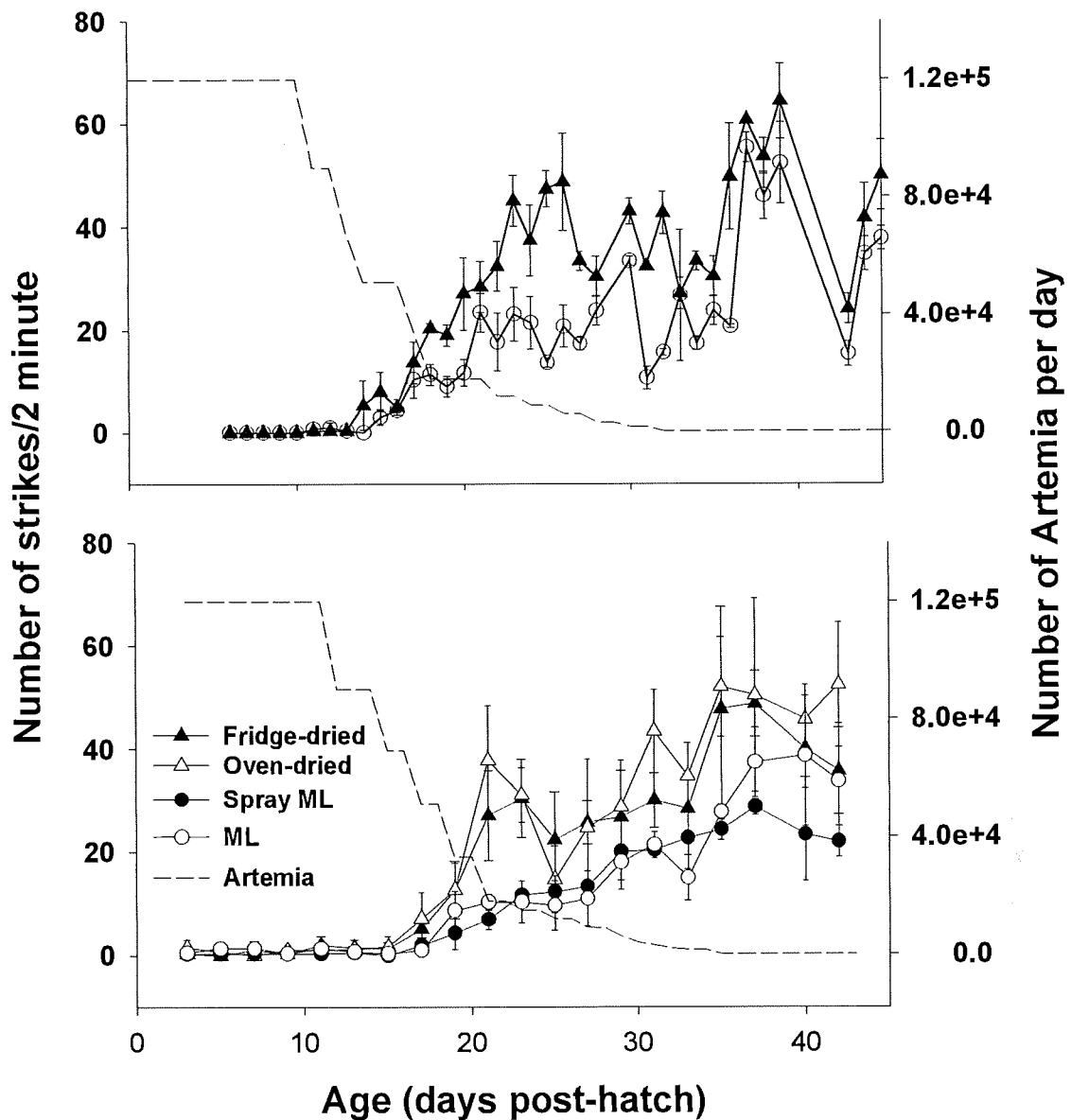
Figure A43:   Strike rate of dhufish larvae on various trial weaning diets.

Although some striking at pellets was observed prior to day 15, strike rates began to increase dramatically after Day 15 in all four groups, when the number of artemia presented was approximately 50,000 nauplii per day (Fig A40). Strike rates in oven and fridge-dried groups appeared to increase more rapidly than both spray ML and ML, although again, statistical analysis was hampered by large variation.

Discussion

Survival in the live feed treatment was similar to the fridge-dried MBD treatment. This was unexpected, particularly as the high strike rates observed in the live feed treatment (data not shown) indicated that the fish were accepting the snapper larvae. Therefore it is possible that the dhufish in this treatment were underfed and the lower than expected survival was due to limitations in food availability not acceptability.

ML was significantly less effective than fridge-dried MBD. This was demonstrated by survival rates and weight at 35 days. Until Day 17, mean strike rate for fridge-dried MBD was always higher than ML suggesting that the difference in survival was due to improved acceptability rather than nutrition advantages.

In contrast to Experiment 1, in Experiment 2, survival of ML fed fish was no different to fridge-dried (or oven dried) MBD. Survival of ML fed fish was approximately 50% in both Experiment 1 and Experiment 2. Survival of fridge-dried MBD fed fish in Experiment 2, however, was approximately half that for Experiment 1. Despite the lack of difference in survival between ML and MBD treatments in Experiment 2, growth was significantly greater in the MBD than ML treatments. Furthermore mean strike rate for ML was generally less than for MBD, indicating that MBD still had positive effect on the acceptance of the pellet. The reason for the lack of difference in survival of larvae fed ML and MBD in Experiment 2 is unknown, however it may be related to the number of times a day the weaning diet was presented. In experiment 1, the weaning diet was presented a maximum of 19 times per day. In experiment 2, due to logistical constraints, the weaning diet in Experiment 2 was only presented 9 times per day. Dhufish are slow, cautious, non-aggressive feeders. It may be that the optimum weaning technique requires constant exposure to the weaning diet. The availability of food in Experiment 2 appears to have compromised the wean. There was no difference in fridge-dried MBD and oven-dried MBD in experiment 2. Since oven-dried MBD is easier to prepare than fridge-dried MBD, oven-dried MBD is selected as the MBD of choice.

Spray ML appears to be the poorest of all treatments as indicated by survival, growth and strike-rate data. The reason for this apparent poor performance of Spray ML is probably largely due to the way the pellet behaved in the water column. The Spray ML pellet was heavier than all the other pellets trialed and so sank to the bottom quickly and did not float in the water column. This may have had a dual effect of reducing the time in which the fish had to strike and also affecting the visual stimuli.

Therefore the use of a microbound diet is a suitable method for weaning dhufish. It is, however, extremely labour intensive. Therefore, a simpler technique must be sort. The wean used in this study was very slow and was conducted on fully metamorphosed fish. Ideally the wean needs to be more rapid and the larvae need to be weaned a younger age.

References

Payne, M., Rippingale, R.J. and Cleary, J.J (2000). Cultured copepods as food for West Australian dhufish *(Glaucosoma hebraicum)* and pink snapper *(Pagrus auratus)* larvae. Aquaculture in press

Appendix O   Evaluation of feed ingredient utilisation and native tissue composition as a precursor to diet development for the West Australian Dhufish (Glaucosoma hebraicum).

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Abstract

The West Australian Dhufish is seen as a potential high-value, endemic species suitable for marine aquaculture in Western Australia. Recent progress with artificial propogation of the species has encouraged further development of grow-out technology, including the development of grow-out diets for the species. To determine the nutritional value of key feed ingredients the digestibility of nine different ingredients were examined. The influence of ingredient inclusion level and the assumption of digestible additivity were also examined. The digestibility of the dry matter of the various meals was highly variable. Protein digestibilities were significantly higher than the dry matter digestibilities for each ingredient. Energy digestibilities were similar to the protein digestibilties for almost all ingredients, except those with high levels of carbohydrates. Dry matter, protein and energy digestibilites were highest for wheat gluten. Protein digestibilities were also high for blood meal, vitamin free casein and high quality fish meal. The native amino and fatty acid compositions of the flesh were also examined to estimate potential essential nutrient requirements. Amino acid composition of the flesh of dhufish was not significantly different to that observed for other species. The native fatty acid composition though was significantly higher in arachidonic (20:4n-6) and docosahexaenoic (22:6n-3) acids, and lower in eicosapentaenoic (20:5n-3) acid than that typically observed in standard commercial fishoils. These nutrient data suggest that standard protein resources, provided digestible limits are observed, will suffice for this species. However, use of fish oil as the only lipid resource may not be sufficient to fully satisfy potential essential fatty acid requirements. Based on the determined digestible protein and energy characteristics of the examined ingredients, and utilising the knowledge gained from the estimated essential nutrient requirements, detailed examination of this species dietary protein and energy requirements is identified as the next required phase of diet development for this species.

Introduction

The West Australian Dhufish is seen as a potential high-value, endemic species suitable for marine aquaculture in Western Australia (Pironet *et al.*, 1999). The species naturally occurs from the Recherche archipelago (S 34° 00', E 122° 00') to Shark Bay (S 26° 00',E 113° 00') in Western Australian coastal waters (Potter and Hesp, 1999). Fishery based data indicates that the fish has a comparatively fast growth rate and has a high current market value (Potter and Hesp, 1999). Anatomically, the fish has characteristics typical of an infrequently feeding carnivore, possessing a large gaping mouth, a relatively large muscular stomach and short gastro-intestinal tract (Halver, 1989).

Development of an effective grow-out diet is seen as one of the key steps in establishing a viable aquaculture industry (Tacon, 1990). Therefore, to expedite diet development for this species it may be prudent to base the requirements for a basal diet on one similar to that required for other carnivorous species such as salmonids and seabass (NRC, 1993).

Effective development of diets for a new potential aquaculture species, such as the dhufish, requires knowledge of the nutrient and energy contributions from various key ingredients used in diet manufacture (Aksnes *et al.*, 1996; Sugiura *et al.*, 1998; Allan *et al.*, 1999). Of particular importance is the identification of the relative level of protein and energy contributions made by each of the ingredients to the diet. Details of these contributions from each ingredient will assist in the next phase of diet development, which is to determine the animal's dietary protein and energy requirements. Consequently this determination of ingredient nutritional value is pivotal to many of the remaining phases of nutritional work required during diet development for a new species (van Barneveld, 1999).

This manuscript reports the examination of the relative digestible dry matter (DDM), digestible protein (DP) and digestible energy (DE) contributions from nine key ingredients. Two further diets examine the complimentary additivity of apparent digestibility values from mixes of several of the key ingredients examined. Also examined is the native amino and fatty acid composition of flesh from wild caught *G. hebraicum*. An assessment of the relative basis of indispensable amino acids (IAA) is made to pre-empt the potential IAA dietary requirements. All these parameters are discussed in context with further development of diets for this species.

Methods

Diet development

A basal diet was formulated and prepared to include approximately 500 g kg^{-1} protein, 150 g kg^{-1} fat and an inert marker (chromic oxide at 4 g kg^{-1}) (Table A23). A basal mash (fishmeal, wheat flour, wheat gluten and fish oil components of the basal diet) was prepared and thoroughly

mixed, forming the basis for all experimental diets in this study. A range of commonly used feed ingredients was obtained from commercial feed companies (Table A24). The ingredient of study for each test diet was sieved to < 500 μm particle size and added to a sub-sample of the basal mash, at a projected final inclusion level of 200 g kg^{-1}. The vitamin pre-mix and marker were added individually to each diet before further mixing (See Table A23). Some ingredients (meat meal, blood meal, and ingredients included in tandem in complementary additivity studies) were added only at 100 g kg^{-1}. Diets were processed by addition of water (about 50% of mash dry weight) to the mash whilst mixing to form a dough, which was subsequently screw pressed using a pasta maker through a 3 mm ∅ die. The resultant moist pellets were then steamed for 4 minutes and oven dried at 80°C for approximately 3 h. The basal diet was prepared in a similar manner, but without the addition of any test ingredient. Composition and source of all of the ingredients used is presented in Table A24.

Fish handling

Hatchery reared juvenile dhufish were obtained from the Aquaculture Development Unit of the South Metropolitan TAFE. The fish were reared from wild-caught broodstock that had been previously acclimated and treated with LHRHa to stimulate ovulation. The larvae and juveniles were reared according to Chapter 6 and 7, this volume.

Eighteen 170 L cylindro-conical settlement-tanks were stocked with 16 to 20 juvenile (7 month, ~ 15 g) Numbers varied depending on experimental block and availability of fish. Treatments were randomly assigned in triplicate to the settlement-tank array. The transferred fish were allowed to acclimate to the settlement-tanks and an allocated dietary treatment for a week before faecal collection commenced. Mortalities were not replaced. Faecal collection was not performed on tanks where a mortality occurred within the preceding 24 h or where feed particles were observed in the settlement tube. Faeces were collected according to the methods described by Allan et al. (1999).

Following acclimation the fish were fed to apparent satiety once daily at about 15:00 hrs (3 PM). Uneaten feed was removed from the collection tube of each settlement-tank following feeding and the feed intake recorded. The cleared collection tube was packed in an iced container and faeces collected by settlement overnight. The settled faeces were collected each morning (08:00) and pooled within tanks and across days (Allan et al., 1999). Faeces were collected over a minimum of six days, and kept frozen (-20°C) pending analysis.

Three blocks (about 14 to 20 days duration) of independent studies were conducted over a seven week period. In each experimental block, one of the six treatments was the basal diet.

Chemical and digestibility analysis

Diet and faecal samples were analysed for dry matter, chromium, protein and gross energy content. Dry matter was calculated by gravimetric analysis following oven drying at 100°C for 24 h. Chromic oxide levels were determined by atomic absorption spectrometry based on the method described by Hillebrand et al. (1953). Protein levels were calculated from the determination of total nitrogen by Kjeldhal analysis, based on N x 6.25. Both of these determinations were conducted according to the methods specified by the AOAC (1984). Gross energy was calculated based on the energetic values of crude protein (23.6 MJ kg^{-1}), fat (39.5 MJ kg^{-1}) and carbohydrate (17.1 MJ kg^{-1}) levels. Differences in the ratios of the dry matter, protein or gross energy to chrome, in the feed and faeces in each treatment were calculated to determine the apparent digestibility coefficient (ADC$_{diet}$) each of the nutritional parameters examined in each diet based on the following formula (Aksnes et al., 1996):

$$ADC_{diet} = 1 - \left(\frac{Cr_{diet} \times Parameter_{feces}}{Cr_{feces} \times Parameter_{diet}} \right)$$

Where Cr$_{diet}$ and Cr$_{feces}$ represent the chromium content of the diet and faeces respectively, and Parameter$_{diet}$ and Parameter$_{feces}$ represent the nutritional parameter of concern (dry matter, protein or energy) content of the diet and feces respectively. Digestibility values for specific ingredients (ADC$_{ingredient}$) in each of the diets were based on the following formula (Aksnes et al., 1996):

$$ADC_{ingredient} = \frac{1}{n} \left(\frac{TD\ Cr_{diet} \times TD\ Parameter_{feces}}{TD\ Cr_{feces} \times TD\ Parameter_{diet}} \right) - \frac{1-n}{n} \left(\frac{BD\ Cr_{diet} \times BD\ Parameter_{feces}}{BD\ Cr_{feces} \times BD\ Parameter_{diet}} \right)$$

Where TD Cr$_{diet}$ and TD Cr$_{feces}$ represent the chromium content of the diet and feces of the test diet respectively, and similarly TD Parameter$_{diet}$ and TD Parameter$_{feces}$ represent the nutritional parameter of concern (dry matter, protein or energy) content of the diet and feces of the test diet respectively. Accordingly the BD notation precedes all specifications concerned with the basal diet.

Samples of wild fish tissue (muscle) were analysed for both their amino acid and fatty acid compositions. The amino acid composition of the flesh was determined with high performance liquid chromatography, using ion-exchange column separation and UV/Vis detection of derivatised amino acids. Fatty acid composition was determined using gas chromatography following trans-esterification of total lipids extracted from the muscle tissue by the method of Folch et al. (1957). Both methods were in accordance with AOAC (1984) specifications.

Statistical analysis

All figures are mean ± SEM unless otherwise specified. Effects of ingredient inclusion on digestibility of dry matter, protein and gross energy were examined by ANOVA. Levels of significance were determined using Tukeys HSD test, with critical limits being set at P < 0.05.

Results

Dry matter digestibilities for all test ingredients varied considerably (Table A25). Highest dry matter digestibility was observed with wheat gluten (0.999) with essentially 100% digestibility. Lowest dry matter digestibility was observed with wheat flour (0.351). Dry matter digestibilities generally showed higher levels of variance in their determination than both protein and energy digestibilties.

Protein digestibilities also varied considerably between ingredients (Table A25). Highest protein digestibility was also observed with wheat gluten (0.999), though this was not significantly higher than that observed with either blood meal (0.987) or vitamin free casein (0.968). In all cases the digestibility of dietary protein was higher than that of respective dry matter.

Energy digestibilities of each of the diets and ingredients were largely consistent with the observed protein digestibilities (Table A25). An exception to this was wheat flour, which had a significantly poorer energy digestibility (0.340) than its protein digestibility (0.660).

The level of inclusion (100 g kg^{-1} or 200 g kg^{-1}) of meatmeal in a test diet did not significantly influence the digestibility values determined for dry matter, protein or energy (Table A25).

The additive digestibility of compounded diets, as estimated from the individual digestibility values, was not significantly different from that measured for actual compound diets (Table A26). Though the high levels of variance observed in these determinations do not provide much statistical power for these parameters.

The flesh amino acid composition of dhufish (Table A27) was highest in glutamic acid (glutamate) at 9.37 g kg^{-1} DM, though the highest likely indispensible amino acid (IAA) was lysine at 7.43 g kg^{-1} DM. On a basis proportional to lysine, glutamate was present at 126%, though of the noted IAA, the highest proportion was leucine (70.0%, 9.37 g kg^{-1} DM) and the lowest was cysteine (10.5%, 0.78 g kg^{-1} DM).

The fatty acid composition (Table A27) of the flesh shows high levels (33.4%) of the long-chained polyunsaturated n-3 fatty acid docosahexaenoate (DHA; 22:6n-3). High levels (7.8%) of the long-chained polyunsaturated n-6 fatty acid arachidonate (ARA; 20:4n-6) were also present. Notable by its absence (0.1%) was eicosapentaenoate (EPA; 20:5n-3).

Discussion

The development of grow-out diets for new species needs to satisfy several demands. Prospective aquaculturists will require information that will enable them to request the most appropriate diet specifications (primarily dietary protein and energy requirements). However, a feed miller requires information on the nutritive value of a suite of ingredients to be able to provide diets to the specifications requested, and to do so on a cost-effective basis. Ideally the nutritive value assessment should detail the available amino acids and metabolisable energy from specific ingredients (Masumoto *et al.*, 1996; Rodehutscord and Pfeffer, 1999). However, preliminary to this is the assessment of digestible protein and energy levels (van Barneveld, 1999). The present study details the nutritive value of nine ingredients frequently used by feed millers to produce diets. Also presented are the animal's native amino and fatty acid compositions to allow preliminary estimation of possible essential nutrient requirements.

Assessment of the nutritive value of key feed ingredients

In this study the ingredient assessment method of diet substitution was used to determine the digestible dry matter, protein and energy values of a range of ingredients, ingredient inclusion levels and complementary additivity (Nengas *et al.*, 1995; Asknes *et al.*, 1996; Aksnes and Opstvedt, 1998; Allan *et al.*, 1999).

The dry matter digestibilities for all ingredients varied considerably (Table A25). The high levels of digestibility of ingredients such as wheat gluten (0.999), blood meal (0.946) and vitamin free casein (0.892) are consistent with what has been reported for similar ingredients in other species such as silver perch, *Bidyanus bidyanus* (Allan *et al.*, 1999). The lowest dry matter digestibility was observed for wheat flour (0.351) and canola meal (0.387), consistent with limited carbohydrase activity being present in this species.

Protein digestibilities also varied considerably between ingredients (Table A26). Similar to what was observed for the dry matter digestibilties, protein digestibility was also high with wheat gluten (0.999), blood meal (0.987) and vitamin free casein (0.968). The energy digestibility of each of the ingredients was also largely consistent with the observed protein digestibilities (Table A25). An exception to this was wheat flour, which had a significantly poorer energy digestibility (0.340) than its protein digestibility (0.660). This observation is consistent with the low protein content and high carbohydrate content of the wheat flour (Tables A24 and A25). The values observed in this study for protein and energy digestibilities are consistent with what has been reported for similar ingredients in other species, notably those with carnivorous tendencies such as atlantic salmon, *Salmo salar* and rainbow trout, *Oncorhynchus mykiss* (Asknes and Opstvedt, 1998; Storebakken *et al.*, 1998; Sugiura *et al.*, 1998; Allan *et al.*, 1999). The observation of poor utilisation of the wheat flour is consistent with there being limited digestion of carbohydrates with this ingredient. It would be of interest to examine the digestibility of this

ingredient when the starch content of the wheat flour has been gelatinsed. In its native form, wheat flour provides limited nutritional value to dhufish.

Generally though, the animal protein meals were not consistently better utilised than the plant protein meals. Plant meals such as soy meal, wheat gluten and canola meal all had protein utilisation values equal to that of any of the animal derived meals. However the actual biological value of these ingredients and the metabolic cost associated with their metabolism remains to be determined. Most appropriate methods to determine this would be by a summit dilution style of experiment as used by Booth *et al.* (1999).

Inclusion of an ingredient at two different levels did not significantly influence the digestibility of that ingredient in any of the assessed parameters of dry matter, protein or energy digestibility. This observation is consistent with what has typically been observed with other species, though it has been noted that the variability of the digestibility determinations decreases with increasing test ingredient inclusion (Smith and Tabrett, 1999). However, in this study it should be noted that the influence of inclusion level was only tested with a single ingredient. This observation would gain further confidence if repeated with at least two further ingredients, with at least two inclusion levels each.

Examination of the additivity of the digestibility parameters determined in this study demonstrated that the digestibility of dry matter, protein and energy values for this species are additive, similar to observations with other species (Allan *et al.*, 2000). However, the high levels of variance observed in some of these determinations reduced the statistical power for these parameters (Searcy-Bernal, 1994). Similar to the determinations of inclusion level on the influence of digestibility, further consolidation of the data would be achieved with examination of both additional compound diets and/or the inclusion of more than two test ingredients in a test compound diet.

Estimation of essential nutrient requirement

The examination of the amino acid composition of the flesh of wild caught West Australian dhufish show high levels of similarity to the flesh of other teleosts (Kaushik, 1998). Evaluation of the IAA requirements, as based on the principles of Kaushik (1998), also suggests that as with most other fish species, that the first limiting amino acid of dhufish is also likely to be lysine. The second most required is likely to be leucine followed by isoleucine, based on the relative abundance of the amino acid of the flesh, relative to the amount of lysine. Based on these speculated requirements and the now known digestibilities of various protein resources, diets providing adequate amino acids for growth at various energy levels should be able to be formulated. However, what this study does not take into account, that would have added considerable value, is the relative digestibilities of each of the amino acids from each of the feed

ingredients (Masumoto *et al.*, 1996). This would considerably strengthened the value of the digestibility data from a feed millers perspective, allowing even more cost effective development of feeds for this species. It would be worth revisiting the digestible requirements of this species on a nitrogen retention basis to determine the most precise requirements for the IAA (Forster and Ogata, 1998).

Of all the dietary nutrients an animal ingests, none more so that the fatty acids have a direct bearing on the composition of those same nutrients in the animal's own tissues (Sargent *et al.*, 1993). Accordingly, an examination of the animal's native fatty acids may possibly enable a qualitative estimation of it's essential fatty acid requirements. In the flesh of wild-caught dhufish the fatty acids of note were the long-chained polyunsaturated n-3 fatty acids: docosahexaenoate (DHA; 22:6n-3), eicosapentaenoate (EPA; 20:5n-3) and although uncommon at appreciable levels in marine species, the long-chained polyunsaturated n-6 fatty acid arachidonate (ARA; 20:4n-6) was also present at appreciable levels. These three fatty acids have often been identified as essential fatty acids (EFA) in other aquaculture species (Watanabe, 1982).

The levels of tissue EPA were notable by their relative absence (0.1%) compared with that seen in most other aquaculture species and industrial fish oils (Glencross and Smith, 1997). In contrast the levels of DHA were considerably higher (33.4%) than those observed seen in most other aquaculture species and industrial fish oils. Similarly ARA was also present at appreciable levels (7.8%). These observations suggest that this species requirement for EPA may be low, though that requirements for both DHA and ARA possibly exist and that those for DHA may be quite high. Despite these speculations, determination of the EFA requirements would not be suggested as a priority for diet development for dhufish until the dietary protein and energy requirements have been more clearly defined.

Further diet development for this species

The determination of the dietary protein and energy requirements are suggested as the next crucial phase of diet development for this species. This information would provide valuable details to optimise diets for cost-effective grow-out production of this species. Combined with the ingredient digestibility information, this would also provide the crucial details required by feed millers for cost-effective diet production. However the determination of the protein and energy requirements of this species should be performed on a digestible protein and energy basis to be of most value (Gurure *et al.*, 1995). This process of nutrient requirement evaluation can be determined even more precisely by conducting critical assessment of the animal's requirements for limiting amino acids (primarily lysine as based on data from this study) per unit of metabolisable dietary energy (Kaushik, 1998). Based on the observations of Kaushik (1998) of the higher protein requirements of marine species, such a definitive determination of nutrient requirements may be warranted.

Acknowledgments

References

Aksnes, A., Hjertnes, T. and Opstvedt, J. 1996. Comparison of two assay methods for determination of nutrient and energy digestibility in fish. Aquaculture, 140: 343-359.

Aksnes, A. and Opstvedt, J. 1998. Content of digestible energy in fish feed ingredients determined by the ingredient-substitution method. Aquaculture, 161: 45-53.

Allan, G.L., Rowland, S.J., Parkinson, S., Stone, D.A.J. and Jantrarotai, W. 1999. Nutrient digestibility for silver perch *Bidyanus bidyanus*: development of methods. Aquaculture, 170: 131-145.

Allan, G.L., Parkinson, S., Booth, M.J., Stone, D.A.J. and Jantrarotai, W. 2000. Replacement of fish meal in diets for Australian silver perch *Bidyanus bidyanus*: Digestibility of alternative ingredients. Aquaculture, 186: 293-310.

AOAC (Association of Official Analytical Chemists). 1984. Official Methods of Analysis of the Association of Official Analytical Chemists. 14th edition. Association of Official Analytical Chemists. Washington, DC, USA.

Arnessen, P., Krogdhal, A. and Sundby, A. 1995. Nutrient digestibilities, weight gain and plasma and liver levels of carbohydrates in Atlantic salmon (Salmo salar, L.) fed diets containing oats and maize. Aquaculture Nutrition, 1: 151-158.

Austreng, E. 1978. Digestibility determinations in fish using chromic oxide marking and analysis of contents from different segments of the gastrointestinal tract. Aquaculture, 13: 265-272.

Booth, M.J., Allan, G.L. and Stone, D.A.J. 1999. Utilisation of four agricultural ingredients by silver perch. World Aquaculture Society Annual Meeting, Sydney, Australia, 26th April – 2nd May 1999. pp 83.

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, 497-509.

Glencross, B. D. and Smith, D.M., (1997) Comparison of triacylglycerols, esterified and free fatty acids as neutral lipid sources in the diet of the prawn, *Penaeus monodon.*, Aquaculture, 159: 67-85.

Gurure, R.M., Moccia, R.D. and Atkinson, J.L. 1995. Optimal protein requirements of young Artic charr (Salvelinus alpinus) fed practical diets. Aquaculture Nutrition, 1: 227-234.

Halver, J.E. (Ed.) 1989. Fish Nutrition. New York: Academic Press.

Hanley, F. 1987. The digestibility of foodstuffs and the effects of feeding selectivity on digestibility determinations in tilapia, *Oreochromis niloticus* (L). Aquaculture, 66: 163-179.

Hillebrand, W.F., Lundell, G.E.F., Bright, H.A. and Hoffman, J.I. 1953. Applied Inorganic Analysis. Wiley, New York, USA.

Kaushik, S.J. 1998. Whole body amino acid composition of European seabass (*Dicentrachus labrax*), gilthead seabream (*Sparus aurata*) and turbot (*Psetta maxima*) with an estimation of their IAA requirement profiles. Aquatic Living Resources. 11(5): 355-358.

Masumoto, T., Ruchimat, T., Ito, Y., Hosokawa, H. and Shimeno, S. 1996. Amino acid availability values for several protein sources for yellowtail (*Seriola quinqueradiata*). Aquaculture, 146: 109-119.

Nengas, I., Alexis, M.N., Davies, S.J. and Petichakis, G. 1995. Investigation to determine digestibility coefficients of various raw materials in diets for gilthead seabream, *Sparus aurata* L. Aquaculture Research, 26: 185-194.

Nengas, I., Alexis, M.N. and Davies, S.J. 1999. High inclusion levels of poultry meals and related byproducts in diets for gilthead seabream, *Sparus aurata* L. Aquaculture, 179: 13-23.

NRC (National Research Council). 1993. Nutrient requirements of fish. National Academy Press. Washington. DC. pp 114.

Pironet, F.N., Bosc, F.A. and Jenkins, G.I. 1999. Preliminary trials of hormone-induced production of fertilised eggs in the West Australian dhufish (Glaucosoma hebracium, Richardson, 19845). Asian Fisheries Science 12: 119-132.

Potter, I.C. and Hesp, S.A. 1999. Determination of the biological parameters required for managing the fishery of West Australian dhufish. FRDC Project 96/103. Final report. Murdoch University, Perth Western Australia. pp 48.

Robaina, L., Moyano, F.J., Izquerdo, M.S., Socorro, J., Vergarra, J.M. and Montero, D. 1997. Corn gluten and meat and bone meals as protein sources in diets for gilthead seabream (Sparus aurata): Nutritional and histological implications. Aquaculture 157: 347-359.

Rodehutscord, M. and Pfeffer, E. 1999. Maintenance requirement for digestible energy and efficiency of utilisation of digestible energy for retention in rainbow trout, Oncorhynchus mykiss. Aquaculture, 179: 95-107.

Sargent, J.R., Bell, J.G., Bell, M.V., Henderson, R.J. and Tocher, D.R., 1993. The metabolism of phospholipids and polyunsaturated fatty acids in fish. In: B. Callou and P. Vittelo (Eds), Coastal and Estuarine Studies - Aquaculture: fundamental and applied research., American Geophysical Union, Washington, DC, USA, 103-124.

Searcy-Bernal, R. (1994) Statistical power and aquacultural research. Aquaculture, 127, 371-388.

Smith, D.M. and Tabrett, S.J. 1999. Measurement of nutrient digestibility in penaeid prawns. World Aquaculture Society Annual Meeting, Sydney, Australia, 26th April – 2nd May 1999. pp 706.

Storebakken, T., Kvein, I.S., Shearer, K.D., Grisdale-Helland, B., Helland, S.J. and Berge, G.M. 1998. The apparent digestibility of diets containing fishmeal, soybean meal or bacterial meal fed to Atlantic salmon (Salmo salar): evaluative of different faecal collection methods. Aquaculture, 169: 195-210.

Sugiura, S.H., Dong, F.M., Rathbone, C.K. and Hardy, R.W. 1998. Apparent protein and mineral availabilities in various feed ingredients for salmonid feeds. Aquaculture, 159: 177-202.

Tacon, A.G.J. 1990. Standard Methods : For the nutrition and feeding of farmed fish and shrimp. Argent Laboratories Press. Redmond, Washington, USA.

Van Barneveld, R.J. 1999. Let's not reinvent the wheel – learning from the terrestrial nutrition sector. World Aquaculture Society Annual Meeting, Sydney, Australia, 26th April – 2nd May 1999. pp 786.

Watanabe, T., (1982) Lipid nutrition in fish., Comparative Biochemistry and Physiology, 73B: 3-15.

Tables

Table A23.   Formulation, proximate and digestibility specifications of the basal diet

Ingredient	g kg^{-1}
Fishmeal – Prime	600
Wheat gluten	100
Wheat flour	200
Fish oil	90
Vitamin premix	6
Chromic oxide	4

Measured Nutrient Content	g kg^{-1}
Dry matter	912
Crude protein	481
Crude fat	144
Carbohydrate[1]	197
Ash	90
Gross Energy (MJ kg^{-1})[2]	20.47

Parameter	ADC
Dry matter digestibility	0.958 ± 0.021
Protein digestibility	0.981 ± 0.009
Energy digestibility	0.982 ± 0.009

[1] Carbohydrate calculated by Dry matter – (Protein + Fat + Ash)

[2] Gross energy calculations based on standard energetic values of protein + fat + carbohydrate (NRC, 1993)

Digestibility values are means ± SEM, n ≥ 3. Other values are means of duplicate determinations

Table A24:   Proximate composition of the ingredients used in the test diets. All parameter values are expressed as g kg^{-1} on an as-received basis.

Ingredient	Dry matter	Protein	Fat	Carbohydrate[1]	Ash	Gross Energy
Fish meal – Prime [a]	924	681	95	0	130	20.15
Fish meal - FAQ [a]	912	638	92	0	180	18.73
Wheat gluten [b]	910	762	8	133	7	20.60
Wheat flour [b]	903	150	13	730	6	15.76
Soy Meal [a]	901	450	13	339	70	17.38
Canola meal [c]	888	438	92	299	60	19.13
Meat meal [a]	854	500	79	0	275	14.84
Blood meal [a]	874	763	55	0	20	20.80
Vitamin Free Casein [d]	940	806	35	0	50	21.26

1 Based on dry matter minus protein, ash and fat content. All values are means of duplicate assay determinations.

[a] Sourced from PIVOT Aquaculture Pty Ltd, Cambridge, TAS, Australia

[b] Sourced from WESTON BioProducts Pty Ltd, Henderson, WA, Australia

[c] Sourced from Davison Canola Mills Pty Ltd, Pinjarra, WA, Australia

[d] Sourced from SIGMA Chemical Company, St Louis, MO, USA

Table A25.   **Digestible nutrient contributions of specific ingredients.**

Ingredient	Dry matter	Protein	Energy
Fish meal – Prime	0.848 ± 0.095	0.938 ± 0.029	0.932 ± 0.042
Fish meal - FAQ	0.714 ± 0.317	0.885 ± 0.148	0.876 ± 0.138
Wheat gluten	0.999 ± 0.000	0.999 ± 0.000	0.999 ± 0.000
Wheat flour	0.351 ± 0.042	0.660 ± 0.020	0.340 ± 0.008
Soy Meal	0.694 ± 0.200	0.900 ± 0.006	0.897 ± 0.066
Canola meal	0.387 ± 0.133	0.852 ± 0.027	0.769 ± 0.076
Meat meal	0.510 ± 0.065	0.850 ± 0.047	0.788 ± 0.025
Meat meal (100 g kg^{-1})	0.544 ± 0.144	0.844 ± 0.050	0.805 ± 0.057
Blood meal (100 g kg^{-1})	0.946 ± 0.018	0.987 ± 0.001	0.982 ± 0.001
Vitamin Free Casein	0.892 ± 0.085	0.968 ± 0.034	0.954 ± 0.041

All test ingredients were included in diets at 200 g kg^{-1} unless otherwise specified.

All values are means ± SEM. n = 3.

Table A26.   **Digestible nutrient contributions from compounded ingredient diets**

Diet	Digestible dry matter	Digestible protein	Digestible Energy
Prime + Flour (10 + 10) - Actual	0.804 ± 0.105	0.947 ± 0.028	0.918 ± 0.047
- Expected	0.886	0.945	0.912
Meatmeal + Casein (10 + 10)	0.783 ± 0.257	0.958 ± 0.074	0.906 ± 0.108
- Expected	0.910	0.966	0.961

All values are means ± SEM. n = 3 (except those of the expected values).

Table 27.   Amino and fatty acid compositions of native dhufish flesh

Amino acid	g kg^{-1} dry matter	% relative to LYS	Fatty acid	% of total fatty acids
Aspartic acid	6.64 ± 0.30	89.4	14:0	2.4 ± 0.56
Glutamic acid	9.37 ± 0.49	126.1	14:1n-7	0.2 ± 0.07
Serine	3.00 ± 0.13	40.4	16:0	19.9 ± 0.60
Histidine*	2.56 ± 0.07	34.5	16:1n-7	3.3 ± 0.55
Glycine	4.55 ± 0.19	61.2	18:0	9.0 ± 0.48
Threonine*	3.22 ± 0.15	43.3	18:1n-7 + n-9	14.3 ± 0.82
Cysteine*	0.78 ± 0.03	10.5	18:2n-6	1.0 ± 0.12
Alanine	3.98 ± 0.03	53.6	18:3n-3	0.3 ± 0.05
Arginine*	3.09 ± 0.13	41.6	20:0	0.1 ± 0.06
Tyrosine*	2.39 ± 0.10	32.2	20:1n-9 + n-	0.8 ± 0.10
Valine*	3.34 ± 0.03	45.0	20:4n-6	7.8 ± 1.09
Methionine*	2.73 ± 0.27	36.7	20:5n-3 (EPA)	0.1 ± 0.05
Phenylalanine*	2.66 ± 0.02	35.8	22:0	2.4 ± 0.15
Isoleucine*	3.55 ± 0.08	47.8	22:1n-9 + n-	0.2 ± 0.07
Leucine*	5.20 ± 0.04	70.0	22:6n-3	33.4 ± 1.80
Lysine*	7.43 ± 0.07	100.0	24:0	1.7 ± 0.14
Proline	2.97 ± 0.10	40.0		
			Saturates[1]	36.6 ± 1.07
			Monounsatur	20.3 ± 1.24
			Polyunsaturat	43.0 ± 2.23
			n-3	33.8 ± 1.75
			n-6	9.2 ± 1.18

[1]Comprises sum of 14:0, 16:0, 18:0, 20:0, 22:0 and 24:0. [2]Comprises sum of 14:1n-7, 16:1n-7, 18:1n-7, 18:1n-9, 20:1n-9, 20:1n-11, 22:1n-9, 22:1n-11, 24:1n-11. [3]Comprises sum of 18:2n-6, 18:3n-3, 18:4n-3, 20:3n-3, 20:3n-6, 20:4n-3, 20:4n-6, 20:5n-3, 22:5n-3, 22:6n-3. Comprises sum of 18:3n-3, 18:4n-3, 20:3n-3, 20:4n-3, 20:5n-3, 22:5n-3, 22:6n-3; Comprises sum of 18:2n-6, 20:2n-6, 20:3n-6, 20:4n-6. * speculated indespensible amino acids

Appendix P   Treatments for ectoparasites and diseases in captive Western Australian dhufish

Authors F. Pironet and J.B Jones

Abstract.

The Western Australian dhufish (Glaucosoma hebraicum), an open-water marine finfish, has been identified as a potential species for aquaculture and a 4 year research project has concentrated on broodstock collection and maintenance, spawning and larval rearing. This paper describes treatments that were developed for the ectoparasites and diseases of broodstock fish. These included bacterial and fungal infections *Cryptocaryon irritans, Haliotrema* sp., an unidentified axinid monogenean, the isopod *Aega cyclops* and copepod *Caligus* sp. Treatments based on betadine, formalin, freshwater, malachite green, oxytetracycline (terramycin), 2-phenoxyethanol, potassium permanganate and trichlorphon (neguvon) were all tried. The most effective treatments against parasites were a freshwater bath or a combined freshwater bath and anaesthesia with 2-phendxyethanol. Monogeneans on the gills were difficult to control and exophthalmia was an unresolved problem.

Introduction

The Western Australian dhufish *(Glaucosoma hebraicum)* (Family Glaucosomatidae), is found in Western Australian waters between Beagle Island (21" 12'S, 115" 30'E) in the Dampier Archipelago in the north, and the Recherche Archipelago (33" 52/S, 121" 54'E) to the south at depths from 10 to -200 m. Highly prized as a game and food fish in coastal areas, this species attains a maximum size of 1.2 m TL and 26 kg (Kailola *et al.,* 1993; McKay, 1997).

Preliminary estimates were that dhufish grow to about 30 cm TL after 1 y and 40-45 cm TL after 2 y (Kailola *et al.,* 1993). Because of its growth potential, the availability of broodstock locally and its high value as a food fish, the dhufish was selected by the Fremantle Maritime Centre (Western Australia) for a research project to establish a breeding protocol in captivity and to assess the viability of this species in aquaculture (Pironet and Neira, 1998).

Dhufish have not previously been bred in captivity and there are no published accounts of diseases and treatments in captive fish. The difficulties encountered in catching and keeping broodstock ensured that each live fish was very valuable which, together with ethical considerations, required that sick fish be treated and restored to health when practicable. Neither the broodstock fish nor their progeny were intended for human consumption and

therefore the use of unregistered drug treatments was permissible. This paper describes the treatments which were applied to control the ectoparasitic fauna and diseases observed in captive dhufish broodstock during the first 2 y of the research project.

Materials and methods

Capture of broodstock fish

A total of 82 dhufish were captured between 1995 and 1997 by hand-line off Ledge Point (31" 7'S, 115" 22'E) in depths of <25 m. Fish caught from waters deeper than 25 m seldom survive due to the effects of decompression (Ashby, unpublished thesis results, University of Western Australia). Upon capture the swim bladder was vented with a sterile 16 gauge hypodermic needle before transport to the Fremantle Maritime Centre in a 900 L aerated tank. On arrival, fish were anaesthetised in 2-phenoxyethanol (approximately 300 $mg.L^{-1}$) and treated to remove external parasites as described below.

Fish were kept in enclosed tanks of 10~50 m^3 capacity, with continuously flowing sand-filtered seawater at a minimum of 10% renewal per hour. Holding tanks were partly covered with black plastic to reduce light intensity. The size of captured fish varied from 1.6 to 12 kg and fish biomass ranged from 0.5 to 3.0 kg/m^3.

Treatments (against skin and gill parasites)

All treatments are summarised in Table A28. Details on some of them are described below.

Phenoxyethanol and freshwater

Fish were sedated in approximately 300 $mg.L^{-1}$ 2-phenoxyethanol, transferred into a 600 L container of freshwater and left for 1.5 h with pure oxygen aeration. Fish were released directly into the holding tank on completion of the treatment. Freshwater used in treatments was potable tap water from the municipal supply (a variable mixture of catchment and bore hole water chemically treated to comply with health standards).

Freshwater

Captive fish requiring treatment were starved for 24 h to avoid induction of vomiting (a common stress reaction in dhufish). Water salinity was lowered to ≤ 1‰ within 1-2 h, maintained at this concentration for 1.5 h, then rapidly raised back to full strength (35 ‰). Salinity was measured with a prism refractometer (0-10 ‰). The temperature difference between the fresh water used in the treatment and the ambient seawater in the tank was not allowed to exceed 2 °C since sudden changes in temperature can be lethal to marine fish (Wedemeyer et al., 1976). Tanks were successively treated in order to achieve three consecutive treatments per tank at 10 day

intervals in September-October (Austral spring) while seawater temperature was -19°C and rising (annual range 15-26 "C). The course of treatments was completed at least 1 month before the beginning of the spawning season (November to April). If mortality of fish occurred at the beginning of a treatment course, freshwater and 2-phenoxyethanol were used in the subsequent treatments.

Freshwater and antibiotic

During the first and tenth day of the treatment course, salinity was lowered to <1‰ within 1 h. Terramycin (10% oxytetracycline active ingredient) was added to reach a final concentration of 300 mg.L^{-1} terramycin and the bath treatment lasted for 1.5 h after which salinity was restored to 359bo by rapid flushing. During the following 8 days, terramycin was administered orally (0.5 g terramycin kg-L body weight). Non-feeding fish were treated instead with a terramycin bath at 300 mg.L^{-1} concentration (as above).

Formalin and malachite green

Fish were treated by lowering salinity to 17~0 then adding formalin and malachite green to reach final concentrations of 75 and 0.24 mg.L^{-1} respectively. Treatment continued for 2.5 h. Treatment was performed at a water level 10 cm above normal level to ensure that the tank "splash zone" was treated. After 2.2 h the water level was lowered to half volume and treatment ended with rapid flushing of the tank with full-strength seawater for 20 min.

Betadine (povodine-iodine) and 2-phenoxyethanol

Fish were anaesthetized in 300 mg.L^{-1} 2-phenoxyethanol for 10-15 min. Lesions were swabbed with Betadine (iodine 1% w/w) and allowed to dry for 10 s before the fish were returned to seawater.

Identification of parasites

Samples of tissue for histology were fixed in 10% seawater buffered formalin, dehydrated in an alcohol series, embedded in wax and stained with haematoxylin and eosin using standard laboratory techniques. Helminths were fixed in Berland's fluid (19 parts pure glacial acetic acid + i part formalin) and stored in 70% alcohol. Crustaceans were fixed in 108 seawater buffered formalin and stored in 70% alcohol prior to identification. If bacterial infection was suspected blood and tissue samples were submitted to the Agriculture WA Animal Health Laboratories for bacterial culture. Voucher specimens of the isopod, copepod and monogenean parasites have been deposited at the Western Australian Museum.

Table A28:   Treatments used, their duration and survival rate of dhufish in captivity at Fremantle

Treatment	Number of fish	Concentration used	Duration of treatment	Survival (%)	Results
2-phenoxyethanol and freshwater	115	300mg.l^{-1} 2-phenoxyethanol	1.5h	100	Effective against isopods, copepods and monogeneans. Not tolerated by spawning fish
Freshwater only	82	<1‰ salinity	Salinity lowered over 1-2h, maintained 1.5h	91.5	Effective against isopods, copepods and monogeneans. Not tolerated by spawning fish
Freshwater and terramycin	6	<1‰ water 300mg.l^{-1} terramycin	Lowered over 1-2h, maintained 1.5h, repeated day 1 and 10	100	Effective in treating monogeneans and concurrent bacterial infections on gills. See text for details.
Formalin and malachite green	24	Salinity 17‰ Formalin 75 mg.l^{-1} Malachite green 0.24 mg.l^{-1}	2.2h	91.7	Effective in treating *Cryptocaryon* Partially effective agaist monogeneans Cannot be used on spawning fish
Betadine + 2 phenoxyethanol	8	300mg.l^{-1} phenoxyethanol betadine swab	Not applicable	100	Very effective on superficial fungal infections
Formalin bath	4	150mg.l^{-1}	1.0h	100	Ineffective on monogeneans
Neguvon*	15	1.1 mg.l^{-1}	5.0h	100	Ineffective on monogeneans
	4	1.25 mg.l^{-1}	15h	50	Partially effective on monogeneans
Potassium permangenate	6	4.2 mg.l^{-1}	1.0h	0	Lethal to fish

Results

Survival after treatments

Results are shown in Table 1. Once the methodology associated with capture and transport of fish was established, including venting, survival was 95%. Newly captured broodstock fish treated with 2-phenoxyethanol and freshwater had 100% survival, though food vomiting was frequent in wild-caught fish. During a freshwater ($\Box$I ‰ salinity) treatment fish typically remained : motionless on the bottom of the container, often lying on their side. A violent swimming reaction preceded death of the fish concerned if its removal from the bath was not immediate.

Ectoparasites and diseases

Bacteria

Bacterial infection appeared as one or several yellow or white-coloured focal gill lesions of up 1 cm^2 in area on 11/82 fish and on two occasions affected approximately 50% of the visible gill area. In six of the eleven cases, these bacteria were identified from blood samples as *Vibrio parahaemolyticus* (dominant species) of concurrent *V. parahaemolyticus* and *V. alginolyticus*. At different times, from three moribund fish without gill lesions uncharacterised *Photobacterium* spp., *P. damsela damsela*, or *V. fluvialis* with unidentified *Vibrio* spp. were isolated from kidney and spleen.

Fungi

Two types of lesions were observed in 8/82 fish, both lesions were associated with the presence of unidentified septate fungal hyphae: a white-coloured lesion on the dorsal surface or caudal peduncle of the fish and a reddish, non-haemorrhagic, calloused abrasion caused by rubbing on the bottom of the tank. A single treatment using 2-phenoxyethanol and Betadine, carried out 2 weeks from first observance of this condition, induced rapid healing of both types of lesion.

Cryptocaryon irritans BROWN, 1951

In August 1996, all six fish in one tank began feeding as normal on day 2 after capture but stopped feeding on day 8. Within 2 weeks vertical stripe like lesions appeared on the Banks of the fish. Lesions were haemorrhagic and oedematous, with scales lifting and shedding. Pyriform anteriorly-pointed theronts and ovoid trophonts were identified as those of *C. irritans* in skin scrapes. Secondary bacterial infections consisting mainly of Vibrio spp and unidentified gram-negative rods were isolated from lesions. One week from first observation of lesions, one fish died. Treatment of the five surviving fish with formalin and malachite green was carried out and repeated twice at 5 day intervals. Lesions healed slowly and disappeared within 3 weeks.

Haliotrema sp.

Flukes of an undescribed species of *Haliotrema* were frequently observed attached to the gills and to the skin over the whole body surface of the dhufish. Flukes measured 50-1100 $\square$m (753 [± 116] $\square$m SD, n = 30) length *in vivo*. Affected fish rubbed with brief tail flicks on the tank bottom or piping. Fish did not appear to be affected by this parasite during the winter period but in September, as the incoming seawater temperature increased to 18"C, they began to display the signs of infection. Examination of skin scrapes confirmed the nature of the parasite. These flukes seemed sensitive to 2-phenoxyethanol, as after 10 min. in approximately 300 mg.L^{-1} concentration 2phenoxyethanol, the majority of the flukes tended to lose grip of their host.

Monogenea: Axininae

A monogenean belonging to the subfamily Axininae was typically observed in low numbers on gills of wild caught dhufish. Flukes multiplied on gills of fish maintained under stressful conditions such as high water temperatures (above 25"C), poor water quality, exophthalmia or overcrowding. Infested fish were dark in colour, displayed frequent opercular movements; dysnopea and mucous production were evident. All freshwater based treatments provided effective control.

Aega cyclops HASWELL, 1881

These isopods were commonly observed on wild fish and typically emerged from the fish nostrils during the freshwater treatment following post-capture anaesthesia.

Caligus sp.

An undescribed species of Caligus was commonly observed on wild fish, clinging on the skin over the head area and particularly the cornea of the eyes. These copepods were associated with ulceration and haemorrhage in untreated fish. They were killed by freshwater treatments.

Non-infectious conditions

Exophthalmia

Unilateral or bilateral exophthalmia occurred in 70 and 20% of the broodstock during the first and second year of study respectively and was also observed in dhufish as young as 5.5 months old. Exophthalmia was initially unilateral, the affected eye was haemorrhagic, swollen and in extreme cases the cornea ruptured. Fish ceased feeding and remained stationary near the bottom of the tank with minimal fin movement. In the worst cases, the fish developed a head-down swimming posture and the unaffected eye often developed mild exophthalmia. No parasite was observed in association with dhufish exophthalmia and, in most cases, no bacteria were isolated from

affected fish eyes. In many cases, but not all, exophthalmia in dhufish was observed within 24 h of a violent impact with a hard surface, self-inflicted during a struggle or scare. Following these observations strict preventive measures were taken to limit self-inflicted damage to the eyes and, as a result, the rate of occurrence of exophthalmia decreased but was not eliminated. Gross examination of affected eyes at necropsy, confirmed by histology, showed that the exophthalmic condition was due to retrobulbar accumulation of blood from haemorrhage of blood vessels in the choroidal rete. No effective treatment was found but in the majority of cases, fishes recovered naturally after several weeks and deep tanks (23 m depth) were more effective in aiding recovery. In severe cases, the damaged eyeball was excised under anaesthesia, which resulted in full recovery of the fish within several days.

Discussion

Dhufish appeared to adapt reasonably well to captivity and the breeding programme met with some success (Pironet and Neira, 1998). Of the ectoparasites and diseases encountered during the project, only the monogeneans and exophthalmia caused persistent concern.

Gill monogeneans are common parasites of marine fish in captivity and can be difficult to control, especially in warm water systems. As a result, there are a large number of attempted treatments in the literature (Cone, 1996). In this study, both fluke species were effectively controlled with freshwater ($\square$1‰ salinity) or with a combination of 2-phenoxyethanol and freshwater baths. It is interesting that a freshwater treatment is tolerated by a large marine fish that normally has no exposure to estuarine or freshwater conditions.

Freshwater baths for up to 5 h have been proved effective at controlling monogeneans in marine fish (Kabata, 1985; Seng and Seng, 1992). However, the safety and efficacy of this type of treatment need to be evaluated for each species (Kaneko *et al.*, 1988). Preliminary observations suggested that recently caught dhufish may be more tolerant to low salinity than fish which have spent some time in captivity, and that juveniles less than one year old may not survive freshwater baths (data not presented).

Combined treatment with formalin and malachite green has been extensively used in aquaculture for the treatment or prevention of fungal and parasitic infections in fish since at least the 1930's. Malachite green is also an effective systemic anti-protozoal agent (Alderman, 1985; Clifton-Hadley and Alderman, 1987; Plakas *et al.*, 1996). Malachite green is still used with ornamental fish in Australia, but is a suspected carcinogen and demonstrated teratogen (Meyer and Jorgenson, 1983), and is not registered for use with food fish. The legal status of most commonly used chemicals in aquaculture, including malachite green, is under review in many countries and there is not yet any international consensus over its use (Schnick *et al.*, 1997). The use of malachite green, in particular, has been severely restricted for food fish use in the USA and in the European Union but continues to be used elsewhere. Malachite green has been used mainly in cold water salmonid culture. Toxicity and effectiveness varies both with

temperature and dye batches, so that Alderman (1985) warned that published "safe dosages" should be regarded with caution. In this study, which deals with a warm water species, the dosage used was not lethal to the fish and was effective against *Cryptocaryon itritans*. However, since *C. irritans* is sensitive to low salinity and undergoes cytolysis at salinities below 20‰ (Colomi, 1985), a freshwater bath may have been just as effective in achieving control. Trials to assess the effect of freshwater treatment on local strains of *C. irritans* remain to be carried out.

Formalin at 15~200 mg.L^{-1} for 26 h has been used successfully in marine systems against many parasites, including monogeneans (Kabata, 1985), but it was ineffective at treating dhufish gill parasites at the dose rates used. Marine monogeneans can also be controlled by 50 mg.L^{-1} Dipferex (Neguvon) for 4-5 min followed 7 d later by 30 mg.L^{-1} for 8-16 min. (Kabata, 1985). Varriale *et al.* (1992) found Neguvon effective at removing *Diplectanum aequans* from sea bass (*Dicentrarchus labrax*) in brackish water at concentrations as low as 0. 15 mg.L^{-1} but with minimum exposure times of 48 h. In dhufish, a dose rate of 1.25 mg.L^{-1} Neguvon for 15 h was lethal to the host and only partially effective at removing monogeneans. Potassium permanganate is a popular treatment for monogeneans (Kabata, 1985), usually in freshwater hosts, either at 2 mg.L^{-1} for an indefinite bath or 3-5 mg.L^{-1} for a single application (Allison, 1957; Kabata, 1985). It was toxic to dhufish in seawater, at a concentration within the recommended range for freshwater use. Toxicity is known to vary widely between species (Wellborn, 1969; van Duijn, 1972). For example, 3 mg.L^{-1} has proved to be toxic to striped bass (*Morone saxatilis*) fingerlings in freshwater and at 1.48 mg.L^{-1} in salinities of 15‰ (Reardon and Harrell, 1994).

A large number of treatments have been recommended against fungal infections. Among these, an indefinite bath of 2-phenoxyethanol at 100 mg.L^{-1} has been successfully used to treat fungus-infected fish (Kabata, 1985, van Duijn, 1972). It is likely that the use of 2-phenoxyethanol as a general anaesthetic at the Fremantle Maritime Centre has kept down the incidence of fungal infections. Betadine, as with other iodine-based disinfectants, is commonly used to disinfect fish eggs, but may also be used as a replacement for the tincture of iodine (at 1.0-50‰) which van Duijn (1972) and Kabata (1985) used successfully to remove fungal lesions from fishes.

Eye disorders such as cataracts and exophthalmia are common diseases amongst fishes maintained in captivity. Eye damage in fish, and exophthalmia in particular, can be caused by bacteria, viruses, parasites as well as by nutritional, temperature and hormonal imbalances or malformations (Dukes, 1975; Blasiola, 1987; Bucke, 1998; Bjerkas and Bjorrnestad, 1999). In many cases, the cause of exophthalmia remains unknown (Chawadhary *et al.*, 1978). Diet is unlikely to be responsible for the disease in dhufish since it was balanced and consisted of frozen fish, squid and prawn supplemented with a stable vitamin mix. Gas-bubble disease is widely reported as a causative agent of exophthalmia in finfish (Dukes, 1975; Kulshrestha and Mandal, 1982; Hauck, 1986; Machado *et al.*, 1987), but was not a cause of exophthalmia in dhufish since the water supply to the tanks was de-saturated by passage through degassing

columns and was monitored for supersaturation. It is hypothesised that the exophthalmia is due to malfunction of the oxygen secretion mechanism within the rete mirabile and pseudobranch (Dehadrai, 1966).

Conclusions and recommendations

The experimental aquaculture of dhufish has provided an opportunity to develop specific treatment protocols for common conditions affecting this offshore marine fish. Despite the lack of exposure to low salinity under normal conditions, the fish has proved remarkably tolerant to freshwater treatments and these are now routinely used at Fremantle. The major fish health problem still facing culture is the propensity of the fish to develop exophthalmia. Further work to determine the aetiopathogenesis of the lesion is currently being carried out.

Acknowledgments

References

Alderman, DJ. (1985) Malachite green: a review. Journal of Fish Diseases 8, 289-298.

Allison, R. (1957) Some new results in the treatment of ponds to control some external parasites of fish. The Progressive Fish Culturist 19, 58-63.

Blasiola, G.C. (1987) Ophthaimic disease of fishes. Freshwater and Marine Aquarium 10(7), 4~50.

Bjerkas, E.,and Bj(amestad, E. (1999) Is there a connection between rapid fluctuation in water temperature and cataract development in the Atlantic salmon (*Salmo salar*). Bulletin of the European Association of Fish Pathologists 19, 16~-169.

Bucke, D. (1998) Cataracts in fanned fish - a multidisciplinary initiative for scientific progress: histological techniques for teleost eyes. Bulletin of the European Association of Fish Pathologists 18, 121-123.

Chawadhary, S.K., Srivastava, J.B. and Shamra, J.P. (1978) Occurrence of exophthalmus in murrel *(Ophiocephalus punctatus)*. Indian Journal of Animal Research 12, 51-52.

Clifton-Hadley, R.S. and Alderman, DJ. (1987) The effects of malachite green on proliferative kidney disease. Journal offish Diseases 10, 101-107.

Colomi. A. (1985) Aspects of the biology of *Cryptocaryon irritans* and hypersalinity as a control measure in cultured gilthead sea bream *Sparus aurata*. Diseases of Aquatic Organisms 1, 19-22.

Cone, D.K. (1996) Monogenea (Phylum Platyhelminthes). In: PTK. Woo (ed.), Fish Diseases and Disorders, Vol I. Protozoan and metazoan infections. CAB International. pp. 289-389.

Dehadrai, P.V. (1966) Mechanism of gaseous exophthalmia in the Atlantic cod *Gadus morhua* L. Journal of the Fisheries Research Board of Canada W, 909-914.

Dukes, T.W. (1975) Ophthalmic pathology of fishes. In: WE. Ribelin and G. Migaki (eds.), The Pathology of Fishes. University of Wisconsin Press, Wisconsin, pp. 383-398.

Hauck, A.K. (L986) Gas bubble disease due to helicopter transport of young pink salmon. Transactions of the American Fisheries Society 115, 630-635.

Kabata,Z. (1985) Parasites and Diseases of Fish Cultured in the Tropics. Taylor and Francis, London, 318 pp.

Kailola, PJ., Williams. MJ., Stewart, PC., Reichelt, R.E., McNee, A. and Grieve, C. (1993) Australian Fisheries Resources. Bureau of Resource Sciences, Canberra, 422 pp. Kaneko, JJ., Yamada. R. Brock, J.A. and Nakamura, R.M. (1988) Infection of tilapia, *Oreochromis mossambicus* (Trewavas), by a marine monogenean, *Neobenedenia melleni* (MacCallum, 1927) Yamaffuti, 1%3 in Kaneohe Bay, Hawaii, USA, and its treatment. Journal of Fish Diseases 11, 295-300.

Kulshrestha, A.K. and Mandal PK. (1982) Pathology of gas bubble disease in two airbreathing cattishes (*Clarias batrachus* Linn. and *Hereropneustes fossilis* Bloch.). Aquaculture 27, 13-17.

Machado, J.P., Garling, J. Kevern, N.R., Trapp. A.L. and Bell, G. (1987) Histopathology and the pathogenesis of embolism (gas bubble disease) in rainbow trout (*Salmo gairdneri*). Canadian Journal of Fisheries and Aquatic Sciences 44, 19851994.

McKay, RJ.,(1997) FAO Species Catalogue Volume 17: Pearl fishes of the world (Family Glaucosomatidae). FAO Fisheries Synopsis 125/17, 28 pp.

Meyer, EP., Jorgenson, T.A. (1983) Teratological and other effects of malachite green on development of rainbow trout and rabbits. Transactions of the American Fisheries Society 112, 818-824.

Pironet EN. and Neira, FJ. (1998) Hormone-induced spawning and development of artificially reared larvae of the Western Australian Dhufish, *Glaucosoma hebraicum* (Pisces: Glaucosomatidae). Marine and Freshwater Research 49, 133-142.

Plakas, S.M., El Said, K.R., Stehly, G.R., Gingerich, W.H. and Allen, J.L. (1996) Uptake, tissue distribution and metabolism of malachite green in the channel catfish (*Ictalurus punctatus*). Canadian Journal of Fisheries and Aquatic Science 53, 1427-1433.

Reardon, I.S. and Harrell, R.M. (1994) Effects of varying salinities on the toxicity of potassium permanganate to larval and juvenile striped bass, *Morone saxatilis* (Walbaum). Aquaculture and Fisheries Management 25, 571-578.

Schnick, R.A., Alderman, DJ., Armstrong, R., Le Gouvetlo, R., Ishihara, S., Lacierda, S., Percival, S. and Roth, M. (1997) World wide aquaculture drug and vaccine registration progress. Bulletin of the European Association of Fish Pathologists 17, 251260.

Seng, L.K. and Seng, L.T. (1992) Treatment of cultured golden snapper *Lutjanus johni* Bloch, infected with monogeneans. Aquaculture 106, 1-8.

Varriale, A.M.C., Cecchini, S. and Saroglia, M. (1992) Therapeutic trials against the *Diplectanum aequans* (Monogenea), parasite of sea bass (*Dicentrarchus labrax* L.) in intensive farming. Bulletin of the European Association of Fish Pathologists 12, 204-206.

Van Duijn, C. Jnr. (1972) Diseases of Fishes, 3rd edition. Iliffe Books, London, 309 pp.

Wedemeyer, G.A., Meyer, EP., and Smith, L. (1976) Diseases of Fishes, Book 5: Environmental Stress and Fish Disceases. T.F.H. Neptune City, NJ, 192 pp.

Wellborn, T.L. Jr. (1969) The toxicity of nine therapeutic and herbicidal compounds to striped bass. Progressive Fish Culturist 31, 27-32.

Appendix Q   Intellectual Property

There are no intellectual property issues regarding this project.

Appendix R   Staff

Personnel	Position	% of time	Period of involvement
Dr Jennifer Cleary	Principal Investigator (ADU)	100	Jan 1998-Aug 2000
Ms Francois Pironet	Principal Investigator (ADU)	100	Jul 1996-Dec 1997
Mr Greg Jenkins	Project Manager (ADU)	10	Jul 1996-Aug 2000
Mr Francois Bosc	Research Biologist (ADU)	100	Jul 1996-Aug 1997
Mr Anthony Aris	Dhufish Technician (ADU)	100	Jan 1999-Aug 2000
Mr Andrew Hughes	Dhufish Technician (ADU)	100	Jul 1997-Jan 1999
Mr Andrew Hughes	Technician (ADU)	5	Jan 1999-Aug 2000
Mr Gavin Partridge	Research Biologist (ADU)	20	Jun 1998-Aug 2000
Mr Ken Frankish	Hatchery Manager (ADU)	10	Jul 1996-Aug 2000
Mr Bruce Ginbey	Assistant Hatchery Manager (ADU)	5	Jul 1996-Aug 2000
Mr Aaron Strawbridge	Technician (ADU)	5	Jul 1996-Aug 2000
Mr Craig Poller	Technician (ADU)	5	Jul 1996-Aug 2000
Mr Dean Kennerly	Technician (ADU)	5	Jul 1996-Aug 2000
Mr James Garde	Technician (ADU)	5	Jul 1996-May 1999
Dr Brian Jones	Fish Pathologist (FWA)	5	Jul 1996-Aug 2000
Dr Sagiv Kolkovski	Research Scientist (FWA)	5	Jul 1999-Aug 2000
Dr Greg Maguire	Research Supervisor (FWA)	5	Jul 1999-Aug 2000
Dr Brett Glencross	Research Scientist (FWA)	5	Jul 1999-Aug 2000
Mr John Curnow	Technician (FWA)	5	Jul 1999-Aug 2000