Novel methods of improving the palatability of feeds containing praziquantel for commercially cultured yellowtail kingfish

By

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A thesis submitted in partial fulfilment of the requirements for the degree of Bachelor of Science with Honours
Declaration

I declare this thesis is my own account of my research and contains as its main content work which has not been previously submitted for a degree at any tertiary education institution.

Luke Pilmer
Abstract

Praziquantel is a broad spectrum anthelmintic drug that has been used therapeutically in humans and other animals for over 30 years. The efficacy of praziquantel against polyopisthocotylean and monopisthocotylean monogenean flukes in fish using bath treatments has been well demonstrated, however such treatments are prohibitively expensive for sea cage operations. Int-feed treatments are also effective, however praziquantel is very bitter and its inclusion into fish diets has a negative impact on food intake and therefore effective dosing. This study aimed to investigate several innovative approaches to improve the palatability of feed containing praziquantel for commercially cultured yellowtail kingfish. The study revealed that freshly applying garlic extract to the surface of pellets coated with praziquantel was highly effective at increasing palatability. Incorporation of praziquantel into mash feeds before pellet extrusion, incorporation of praziquantel into hydrogenated castor oil solid lipid nanoparticles and the use of transglutaminase to strengthen the gelatin binder all proved ineffective at increasing palatability. When the garlic extract was applied 5 days prior to feeding the diets, palatability was reduced. Fish fed diets freshly coated with garlic extract consumed 100% of the ration at a praziquantel dietary inclusion level of 5 g/kg. As the praziquantel inclusion level increased to 15 g/kg the consumption reduced slightly, but not significantly. The time taken to consume the ration was significantly slower in diets containing praziquantel and garlic extract coating compared to diets without praziquantel, regardless of praziquantel dietary inclusion level. This study focused on juvenile kingfish less than 350grams. Future research should revolve around larger kingfish, testing lower concentrations of garlic extract and testing efficacy of the actual treatment in fish infested with flukes. The findings have considerable potential to increase the efficiency of application of praziquantel to yellowtail kingfish and potentially other cultured finfish.
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Acknowledgments

Firstly, I would like to thank the entire team of my current employer at the Australian Centre of Applied Aquaculture Research for providing me with the time and resources needed for me to complete my studies.

A massive thankyou to both of my supervisors Alan Lymbery and Gavin Partridge. This project would not have been possible without your insight and advice. The fast response to emails and weekend replies has been greatly appreciated.

Finally, I would like to thank my partner Natalie Duncan for help with fish moves, weekend trial work and putting up with my late-night writing sessions to complete this thesis.

If you had told me a year ago that I would be completing my honours I would not have believed you. But upon completion of this work I have found it very rewarding and I have developed some vital skills to develop my scientific career.
1 Introduction

1.1 Aquaculture industry

Aquaculture has been the world’s fastest growing animal food producing sector for the last three decades (Abate et al., 2016) and the aquaculture industry currently supplies half of the world’s human seafood consumption. In 2012, total global aquaculture production reached 90.4 million tonnes, corresponding to a value of US$144 billion (Food & Agriculture Organization of the United Nations, 2014). Aquaculture is an industry that has had immense improvements in productivity, which has allowed it to reduce production costs and pass lower prices onto consumers (Smith et al., 2010). Furthermore, aquaculture production has a smaller carbon footprint than the production of terrestrial animals (Torrissen et al., 2011). Seafood also provides a healthy alternative to other sources of protein as it contains omega 3 and 6 fatty acids (Abate et al., 2016).

Aquaculture is the fastest growing primary industry in Australia (Buckley, 2005). The industry had a gross production value of AUS$870 million in 2009-10 (Australian Bureau of Agricultural and Resource Economics and Sciences, 2010); representing 34% of the total gross value of Australian fisheries production (Australian Bureau of Agricultural and Resource Economics, 2005). The majority of Australia’s aquaculture is based in regional areas, which has significant positive outcomes for regional development (Buckley, 2005).

The earliest commercial aquaculture product in Australia was the Sydney rock oyster in New South Wales in 1872 (Australian Bureau of Agricultural and Resource Economics, 2003). Australia now has 50 different species being produced commercially (Buckley,
2005), although 90% of Australia’s gross production value is derived from just five
species, namely; Atlantic salmon, bluefin tuna, pearls, oysters and prawns (Table 1.1)

Table 1.1 Major species in the Australian aquaculture industry, value and total
production for 2007-2008 (Fisheries Research and Development Corporation and Ridge
Partners 2010)

<table>
<thead>
<tr>
<th>Species</th>
<th>Scientific name</th>
<th>Production amount ( tonnes )</th>
<th>Gross Value of Production ($'000)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atlantic salmon</td>
<td>Salmo salar</td>
<td>25,527</td>
<td>299,259</td>
</tr>
<tr>
<td>Southern bluefin tuna</td>
<td>Thunnus maccoyii</td>
<td>9,757</td>
<td>186,742</td>
</tr>
<tr>
<td>Pearl oysters</td>
<td>Pinctada maxima</td>
<td>na</td>
<td>114,292</td>
</tr>
<tr>
<td>Sydney rock oyster</td>
<td>Saccostrea glomerata</td>
<td>12,460</td>
<td>89,130</td>
</tr>
<tr>
<td>Pacific oyster</td>
<td>Crassostrea gigas</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native flat oyster</td>
<td>Ostrea angasi</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milky or northern oyster</td>
<td>S. amasa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Backlip oyster</td>
<td>S. echinata</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Giant tiger prawn</td>
<td>Penaeus monodon</td>
<td>3,088</td>
<td>44,203</td>
</tr>
<tr>
<td>Banana prawn</td>
<td>P. meruiensis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brown tiger prawn</td>
<td>P. esculentus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kuruma prawn</td>
<td>P. japonicus</td>
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</tr>
</tbody>
</table>

1.2 Yellowtail kingfish (*Seriola lalandi*)

Yellowtail kingfish is a temperate, carnivorous, pelagic, finfish species that has a
circumglobal distribution in subtropical waters (Bowyer et al., 2012). Yellowtail
kingfish is perfectly suited to sea cage culture as it is a fast growing and economically
valuable species (Fernandes, 2008). In Australia, the predominant production of
yellowtail kingfish occurs in South Australia with emerging industries in both New
South Wales and Western Australia (Booth et al., 2010a).

Yellowtail kingfish and related species, *Seriola dumerili* (amberjack) and *Seriola
quinqueradiata* (Japanese yellowtail) support commercial and recreational fisheries
worldwide, and a significant aquaculture industry in Japan (Nakada, 2002). This
industry largely relies on capture and on-growing of wild juveniles in sea cages, although there is some production of commercially cultured larvae and juveniles (Poortenaar et al., 2001). In the Australian yellowtail kingfish industry, all the fish come from closed-cycle hatchery production (Miller et al., 2011). Yellowtail kingfish is a premium quality product and is marketed as whole fish or fresh and frozen fillets, cutlets and loins. In the Japanese sashimi market yellowtail kingfish is an extremely valuable product, second only to tuna (Whatmore et al., 2013). However, with steadily reducing catches and quotas for bluefin tuna (Thunnus maccuroi and Thunnus orientalis), the value and demand for yellowtail kingfish will continue to grow (Whatmore et al., 2013). The increasing demand for yellowtail kingfish heightens the importance of new technologies to maximise the growth and production of this species through nutrition and disease management.

The current production of yellowtail kingfish in Australia is close to 4000 tons a year with an annual market value of $60 million/year (Booth et al., 2010a). Yellowtail kingfish is expected to have continued growth, with a predicted increase of production of 7% per year until 2020 (Figure 1.1)
Although the aquaculture industry is growing, its growth is being constrained by several factors, including aquaculture legislation, aquaculture zoning, environmental factors and parasite or disease issues. A key constraint to all aquaculture growth is legislation. For example, the Western Australian government has created a new aquaculture zone in the Midwest region to promote aquaculture. This new zone is dedicated for marine finfish native to Western Australia’s West Coast Bioregion, including yellowtail kingfish, mahi mahi (*Coryphaena hippurus*), snapper (*Pagrus auratus*), mulloway (*Argyrosomus japonicas*), coral trout (*Plectropomus leopardus*) and various cod and tropical snapper species. The maximum limit for the zone is proposed to be 24,000 tonnes of finfish (Department of Fisheries Western Australia, 2015).

**1.3 Disease management in aquaculture**

Another key constraint to the aquaculture industry is disease (Sheppard, 2005). Disease can limit aquaculture productivity in several ways. Firstly, the direct cost due to disease...
induced fish death. Secondly, this loss is magnified by an elevated cost-of-production due to sub-optimal growth and poor feed conversion. Thirdly, some disease agents can affect seafood quality and therefore marketing. In addition, pathogens are frequently used as non-tariff barriers to trade in seafood products. Finally, international experience has shown that the public expresses deep concern about disease in semi-open aquaculture settings. Concerns about the use of drugs and chemicals to control diseases, the perception of food safety, and worries about loading the environment with farm-magnified pathogens have all plagued the international fish farm industry (Sheppard, 2005).

There is at least 26 documented disease that affect commercially cultured species of *Seriola*, including viruses, bacteria, parasites and physical deformities (Sheppard, 2004). One of the most concerning diseases of cultured yellowtail kingfish is parasites, as they often develop higher parasitic burdens than wild fish, as fish-farming conditions promote infection by some parasite species (Sánchez-García et al., 2014).

There are several reasons why parasitic disease is more prevalent in cultured than in wild fish populations. Firstly, cultured fish are kept at much higher densities than seen in the wild, which increases parasite transmission rate (Seng, 1997). This enhanced transmission is exacerbated by the inability of fish to move away from sources of parasites to other areas (Williams, 2010). Secondly, the production of marine finfish requires handling, treatment and movement of the fish, all of which are important stressors (Conte, 2004). This, in combination with crowding and a reduction in water quality, may reduce immunocompetence and thus increase the animal’s susceptibility to parasitic disease (Conte, 2004). Thirdly, the majority of aquaculture production
systems, especially in the marine environment, revolve around open water cage production, which exposes cultured fish to parasite transmission from wild fish on a regular basis and limits control over environmental conditions, such as temperature and salinity (Whatmore et al., 2013).

Recent research indicates that up to 40 metazoan parasite species can infect wild yellowtail kingfish in southern and eastern Australian waters (Hutson et al., 2007). Currently however, only two parasite species require active management in the yellowtail kingfish industry, the monogeneans *Benedenia seriolae* and *Zeuxapta seriolae*.

### 1.4 Monogenean parasites

Monogeneans are a group of metazoan platyhelminth parasites (Phylum Platyhelminthes, Class Monogenea) that are recognised as a significant constraint to finfish aquaculture (Abidi et al., 2011). Monogeneans have been regularly cited as causes of reduced growth and survival within farmed fish (Thoney and Hargis Jr, 1991, Abidi et al., 2011). With the tremendous growth of the aquaculture industry in the last 20 years, there has been a dramatic increase in the awareness of monogeneans as pathogenic organisms (Whittington and Chisholm, 2008).

Monogeneans are usually found on the external surfaces of the host, where they attach via hooks called a haptor. They are found in areas around the eyes, gills, fins and buccal cavity (Thoney and Hargis Jr, 1991). Monogeneans can be divided into two sub classes based on the morphology of the attachment organ; the Monopisthocotylea and the Polyopisthocotylea. Monopisthocotylea have a simple feeding organ that they utilise
when feeding on the skin and fins of their hosts as well as to attach to the epithelial surface (Whittington and Chisholm, 2008). Polyopisthocotylea have a complex haptor that is made up of several components (Thoney and Hargis Jr, 1991); these parasites feed on host blood and are usually found only on the gills.

There are four main species of monogenean parasites that can affect yellowtail kingfish production; *Benedenia seriolae, Neobenedenia sp., Heteraxine heterocerca* and *Zeuxapta seriolae*. Of these, *Z. seriolae* and *B. seriolae* are the two most prominent and detrimental parasites in Australia (Sharp et al., 2004).

*Zeuxapta seriolae* is a polypisthocotylean monogenean that attaches to the gill lamellae using multiple haptoral clamps and feeds on blood (Mooney et al., 2006). Infestations of *Z. seriolae* have caused anaemia, reduced appetite and decreased growth in yellowtail kingfish grown in Australia and New Zealand (Mooney et al., 2006).

*Benedenia seriolae* is a monopisthocotylean that inhabits the skin and fins and feeds on mucus and epithelial cells (Chambers and Ernst, 2005). Without regular intervention, *B. seriolae* populations can impact negatively through the loss of fish growth, decreased market value due to parasite induced damage of the fish and fish mortality (Hutson et al., 2007).

### 1.4.1 Pathology

The pathology of monogenean infestations is varied, with the effects changing depending on the family of parasite and host species. Heavy infestations of blood feeding monogeneans can cause anaemia, resulting in direct death of the fish (Kim and Choi, 1998). The host response to monogeneans is nonspecific, involving mainly

1.4.2 Lifecycle

Monogeneans have a direct lifecycle, which means that no intermediate host is required for the parasite to reproduce (Reed et al., 2012). The time required for maturation from eggs to adults is temperature dependent. At water temperatures of 22-25°C, only a few days may be required for completion of the life cycle, where at lower temperatures (1-2 °C), generation time may be extended to five or six months (Reed et al., 2012).

Transmission of monogeneans from fish to fish can occur through direct contact or through ciliated larvae hatching from eggs that are released into the environment (Reed et al., 20012). Once immature monogeneans find a host, they crawl on the surface of the host’s body to reach the preferred position (gills, head etc.). This single host lifecycle means that in intensive farming situations, the monogenean burden is quickly transmitted between hosts and population numbers can increase rapidly (Thoney and Hargis Jr, 1991). Furthermore, eggs from monogeneans attach to sea cages increasing the chance and rate of reinfection (Williams, 2010).

1.5 Treatment of parasites

Chemical treatment is the principal method used to control parasites in the aquaculture industry. The most commonly used chemicals to treat infections are sodium chloride (in freshwater fish), formaldehyde, copper sulfate, hydrogen peroxide and anthelmintic drugs such as mebendazole, trichlorphon and praziquantel (Eiras et al., 2008). These
therapies have two major administration methods being bathing in the chemicals or oral medication. The type of treatment is determined by the species of parasite that is being targeted, the sensitivities of the host and the logistical considerations depending on the type of farming operations (Eiras et al., 2008).

1.5.1 Bathing

Bathing is achieved by completely enclosing the area where the fish are held, through either enclosing the fish in a tarpaulin, or by physically removing the fish from the sea cage. Several studies have investigated various bathing treatments for the control of parasites (Burridge et al., 2010). One of the most common methods of treating parasites is the use of dechlorinated freshwater (to treat marine fish) and saltwater (to treat freshwater fish). These treatments are not restricted by drug legislation and there are no withholding periods (Eiras et al., 2008). This method is effective at removing skin parasites but is less effective at removing gill parasites (Thoney and Hargis, 1991).

Avermectins are effective in the control of internal and external parasites in a wide range of host species and have been used to treat sea lice in salmon at an effective dose of 0.05 mg L\(^{-1}\) fish per day for seven consecutive days. (Burridge et al., 2010). Sea lice may also be treated by a one hour bath in the synthetic pyrethroids cypermethrin or deltamethrin at a concentration of 5.0 µgL\(^{-1}\) (Burridge et al., 2010). Hydrogen peroxide, a strong oxidizing agent, is also widely used for parasite infections of fish in hatcheries; the recommended concentration for bathing treatments is up to 300 mgL\(^{-1}\) for 30 mins every 2 days (Eiras et al., 2008).

The current and most widely used method of treating monogenean infestation in yellowtail kingfish is through bathing the fish in hydrogen peroxide or fresh water
Fish must be bathed within a strict concentration to ensure effective treatment of parasites if hydrogen peroxide is used. Bathing is labour intensive, time consuming, weather dependant and prolonged exposure can detrimentally impact the fish, causing reduced growth rates and in some cases mortalities (Gaikowski et al., 1999). Bathing treatments are also expensive; in Japan, for example, the cost of bathing to treat *B. seriolae* contributes up to 22% of the production costs of sea caged *Seriola* species (Ernst et al., 2002). Constant handling, crowding, loss of feeding time and reductions in dissolved oxygen during bath treatments can cause mortalities as well as diminished appetite and loss of growth (Grant, 2002). In addition, bathing treatments provide only partial control of monogenean populations in farmed fish as monogenean eggs are resistant to chemical treatments (Sharp et al. 2004). There is, therefore, an urgent need to investigate treatment methods that can break the monogenean lifecycle (Sharp et al., 2004).

1.5.2 Oral administration

Oral administration of medications to sea cage fish has advantages over conventional bath treatments. Overall, the inclusion of oral treatments requires less labour and time, as well as less stress for the fish (Grant, 2002).

In-feed medications have wider safety margins and do not require crowding or increased handling of fish. Treatment efficiency is also increased, as all cages on the farm can be treated quickly (Williams et al., 2007) reducing the chance of infection from nearby untreated fish. Through feeding medicated diets, the fish can also maintain their natural feeding regime, also reducing stress (Conte, 2004). Environmental impact of oral treatments are less than bathing treatments, as once the bathing process is
finished, the chemicals are released into the surrounding environment, which may impact non-target organisms (Grant, 2002). Oral treatments are eventually released into the environment, but at much lower concentrations and at a much slower rate (Ramstad et al., 2002).

Oral feeds do also have some disadvantages compared to bathing treatments. As the drug dosage is given uniformly across the feed, differences in sizes and feeding habits of the fish can result in the drug not being delivered in effective doses to some of the less voracious or smaller fish (Shet and Vaidya, 2013). Chemical treatments can also detrimentally impact the palatability of the feed, which can cause reduced feeding, thereby reducing the dose of the medication that is received meaning that the medication may be ineffective (Williams et al., 2007).

1.6 The use of praziquantel in aquaculture

Praziquantel is a pyrazinoisoquinoline anthelmintic that was released to the human medical market in 1975 specifically to target platyhelminths (Harder, 2002). Praziquantel is used to treat platyhelminth infections in people, livestock and companion animals (Day et al., 1992). Although an enduring and thoroughly researched anthelmintic, the mode in which praziquantel acts is still to be fully elucidated (Kohler, 2001). It appears to interfere with the calcium flux across the worm’s tegumental membranes, causing vacuolisation and muscle spasms (Greenberg, 2005b). While parasites may not be killed directly, they ultimately lose their ability to attach.

For fish, praziquantel is typically administered as a bath treatment, at concentrations of 2.5 to 20 mg L^{-1} for 3 to 48 hours (Sharp et al., 2004, Hirazawa et al., 2000a), or orally
at doses of 50 to 400 mg kg\(^{-1}\) daily for up to 20 days (Kim and Choi, 1998a, Hirazawa et al., 2000a). Praziquantel is commercially available to Japanese aquaculturalists as Hadaclean® (Bayer Japan) for the treatment of \textit{B. seriolae} at 150 mg kg\(^{-1}\) daily for 3 days for an oral treatment (the dose recommended on the product label) (Stephens et al., 2003, Tubbs and Tingle, 2006b).

Pharmokinetic studies of praziquantel have been performed in animal and humans using a variety of different approaches (Cioli and Pica-Mattoccia, 1995). Absorption of praziquantel after oral administration is rapid (maximal serum concentration is reached in 1-2 hours) and almost complete (80-100% of the intravenous dose) (Greenberg, 2005a). Drug elimination occurs mainly (80%) in the urine, the rest being found in bile and faeces. These pharmokinetic studies show that parasites are exposed to micro molar concentrations of unchanged praziquantel for a relatively short time.

Orally administered praziquantel is also effective against polyopisthocotylean monogeneans, including \textit{Microcotyle sebastis} parasitising \textit{Sebastes schlegeli} in Korea (Kim and Cho, 2000, Kim and Kim, 2002, Kim and Choi, 1998b), \textit{Heterobothrium okamotoi} parasitising \textit{Takifugu rubripes} in Japan (Hirazawa et al., 2000b), \textit{Zeuxapta seriolae} parasitising farmed \textit{Seriola lalandi} in New Zealand (Tubbs and Tingle, 2006a) and Australia (Williams et al., 2007) and \textit{Sparicotyle chrysophrii} parasitising \textit{Sparus auratus} in the Mediterranean (Sitjà-Bobadilla et al., 2006).

Although effective against monogenean parasites, the oral administration of praziquantel through individual dosing is not logistically viable for large aquaculture operations. Bath treatments, especially at the higher recommended concentrations, are also prohibitively expensive for large sea cages. Having an alternative medicated feed
option for the administration of praziquantel would therefore be a great advancement for the yellowtail kingfish industry.

1.7 Improving the palatability of praziquantel in yellowtail kingfish

The palatability of praziquantel is a well-known limiting factor when the drug is used orally for humans, where the combination of high dose and strong bitter flavour results in lessened usage in developing countries where it is used for treating parasites (Meyer et al., 2009). In veterinary medicine, the oral delivery of praziquantel to taste sensitive companion animals is also known to be a challenge (Oppel, 2008). Within the yellowtail kingfish context, praziquantel’s strong bitterness has resulted in appetite suppression and diet rejection (Partridge et al., 2014). Methods to achieve taste masking are applying medication directly into feed during processing, coating with flavours, binders and encapsulation. (Oppel, 2008).

1.7.1 In-feed

Having the ability to purchase medicated feed diets that are ready for farm use would likely be beneficial to the aquaculture industry. Currently medication being added into the feed during the processing process is not a popular method to treat fish, as it is not cost effective and can be time consuming. If a method can be developed that is guaranteed to increase palatability of praziquantel, this may encourage some feed producers to adopt in-feed medication. In-feed medication has been previously used to increase the palatability of antibiotics in seabass (Dicentrarchus labrax); this process also reduced leaching of the medication, meaning more medication was consumed (Rigos et al., 1999). This is encouraging as adding praziquantel to feed may be a simplified way to increase its palatability. However, while Williams et al (2007)
proposed that incorporating praziquantel into feed mash prior to extrusion may increase palatability, Partridge et al (2014) tested this theory and found no clear benefit.

1.7.2 Flavours

Currently, when medicated diets are used, the most common method of increasing palatability is using flavour masking agents or feed enhancers. A multitude of flavour masking and scent covering materials have been trialled to increase palatability of feeds that contain unusual ingredients, or medicated diets. These include masking scent and flavour with fish oils (Kubitza et al., 1997, Partridge et al., 2012). Partridge et al (2012) tested five different flavour masking agents and found some benefit in surface coating diets with flavours, although diets were not 100% consumed. Anecdotal evidence from Japan indicates that garlic can be used as a masking agent for praziquantel in *Seriola*, however there appears to be no published data on this.

1.7.3 Binders

Binders such as gelatin are commonly used to adhere medication to feed in the aquaculture industry. Partridge et al (2012) tested four different binder types and combinations at different concentrations, with gelatin performing the best. Transglutaminase produces inter- and/or intra-molecular bonds between γ-carboxamides of glutamine residues and ε-amine groups of lysine residues (Kieliszek and Misiewicz, 2014). The formation of intermolecular bonds between proteins by transglutaminase decreases gelatin film solubility (Staroszczyk et al., 2012). Previous studies have shown that transglutaminase and processing conditions can be used to tune the final solubility, mechanical properties and gelation kinetics of gelatin gels by combining different amounts of physical and chemical networks (Liu et al., 2016).
Transglutaminase will increase the strength of the gelatin and therefore may be more effective at masking the flavour of praziquantel.

1.7.4 Microencapsulation and nanoparticles

Microencapsules are coated particles in the micrometer range, with diameters of 1-1000 μm (Singh et al., 2010). Microencapsulation is the process in which small droplets or particles of liquid or solid material are surrounded or coated by a continuous film of polymeric materials. The microencapsulation procedure was discovered by Bungenburg de Jon and Kan in 1931 to help with the preparation of gelatine spheres and use of a gelatine coacervation process (Ankit et al., 2011). The main reason that microencapsulation was developed was to enable a sustained release of drug when needed, however it was found that the process of microencapsulation also resulted in the bitter or unpalatable taste of some medications being reduced (Ankit et al., 2011).

Microencapsulation differs from standard particle coating processes based on the techniques used to obtain the drug microcapsules. The method includes coacervation phase separation, spray drying and congealing, solvent evaporation and multi orifice centrifugation techniques. Microencapsulation technology is being developed for a wide range of unpalatable drugs (Singh et al., 2010).

Microencapsulating praziquantel was used successfully by Partridge et al (2014) to improve the palatability of praziquantel-containing feeds in yellowtail kingfish. However, whilst palatability was improved, these authors found evidence of reduced bioavailability and hypothesised that this was due to the hard coating not being fully digested by the fish, thereby reducing bioavailability. Therefore, these results show
promise for such particles to improve taste, although it is important the bioavailability is not reduced.

Nanoparticles are smaller than microcapsules, ranging in size from 50 to 1000 nm. Their smaller size allows them to be absorbed into the body at much more efficient rates than microcapsules. This means a potential increase in bioavailability of drugs that use this method compared to microencapsulation (Kohane, 2007). Nanoparticles have received considerable attention in the field of drug delivery (Xie et al., 2008). Solid lipid nanoparticles (SLN) introduced in 1991 represent an alternative carrier system to traditional colloidal carriers such as emulsions, liposomes and polymeric micro and nanoparticles (Vijayan et al., 2013). Solid lipid nanoparticles consist of spherical solid lipid particles in the nanometer range, which are dispersed in water or in aqueous surfactant solution (Deshmukh, 2014). They are comprised of a solid hydrophobic core having a monolayer of phospholipid coating. The solid core contains the drug dissolved or dispersed in the solid high melting fat matrix (Deshmukh, 2014). They have the potential to carry lipophilic or hydrophilic drugs such as praziquantel (Vyas and Khar, 2004). In the past decades, SLN have received considerable attention in the field of drug delivery as an alternative dosage form to other colloid nanoparticles (Xie et al., 2008). The main advantages of SLN are that they have good biocompatibility and biodegradability, high bioavailability, offer sustained release, can be produced on a large scale and can be delivered by almost all routes (Xie et al., 2010). The use of SLN in mice has shown an increase in bioavailability of praziquantel by a factor of 10 (Xie et al., 2010). If the same result was found for yellowtail kingfish, then the required dose rate could be reduced by 10 times, therefore increasing palatability. However, while
data are available on increased bioavailability in other animals, there are no published data on bioavailability or palatability for praziquantel incorporated into nanoparticles in fish.

1.8 Aims and hypotheses

The aim of this study was to investigate several innovative approaches to improve the palatability of feed containing praziquantel for commercially cultured yellowtail kingfish.

The hypotheses that were tested are:

1. The inclusion of praziquantel in feed mash prior to extrusion into pellets will increase palatability relative to coating praziquantel onto the surface of the pellets after extrusion.

2. Coating praziquantel medicated feed with garlic extract will increase the palatability to yellowtail kingfish.

3. Praziquantel SLN will increase the palatability of praziquantel medicated feed to yellowtail kingfish.

4. The inclusion of transglutaminase to the gelatin coating of praziquantel medicated feed will increase the palatability to yellowtail kingfish.

2 Materials and Methods

2.1 Experimental design
This study comprised 6 separate trials to test the aforementioned hypotheses. This combination of trials summarized below (Table 2.1) and detailed in Section 2.6, was necessary because of the complexity of the interactions among the taste masking procedures we investigated and the limited number of experimental tanks available for any one trial.

**Table 2.1** Trial numbers, aim for each trial and hypotheses tested

<table>
<thead>
<tr>
<th>Trial</th>
<th>Aim</th>
<th>Hypotheses tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Compare application methods and garlic extract concentration on the palatability of diets containing praziquantel</td>
<td>1 and 2</td>
</tr>
<tr>
<td>2</td>
<td>Determine whether incorporating praziquantel into hydrogenated castor oil solid-lipid nanoparticles (HCO-SLN) improves palatability of diets containing praziquantel</td>
<td>2 and 3</td>
</tr>
<tr>
<td>3</td>
<td>Determine whether freshly applying garlic extract to diets containing praziquantel improves palatability relative to those coated 5 days prior to use</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>Determine whether incorporating praziquantel into chitosan coated HCO-SLN improves palatability of diets containing praziquantel</td>
<td>2 and 3</td>
</tr>
<tr>
<td>5</td>
<td>Determine whether strengthening the gelatin binder improves palatability of diets containing praziquantel</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>Determine the interactive effect of praziquantel inclusion level and garlic extract concentration on palatability of diets containing praziquantel</td>
<td>2</td>
</tr>
</tbody>
</table>

**2.2 Fish**

Juvenile yellowtail kingfish were sourced from the Australian Center for Applied Aquaculture Research. Prior to each experiment the fish were maintained in one 10m³ tank. In this tank fish were fed daily to satiety with Skretting ‘Nova FF’ 5mm floating pellets (Skretting Australia™). At the end of each trial, fish were returned to a different tank (10m³) to ensure that all fish used in the trials were naive to the taste of praziquantel.
2.3 Treatment preparation

Praziquantel was sourced from The TNN Development Limited, Dalian China

The garlic extract vitamin B1 complex (GB1) was sourced from Fuji-Sangyo Co., LTD, Japan

Praziquantel incorporated nanoparticles were produced at the Ian Wark Research Institute at the University of South Australia. Composition and nanoparticle preparation methods are shown below (Table 2.2). The nanoparticles used were hydrogenated castor oil solid lipid nanoparticles (HCO-SLN). This type of nanoparticle was selected over alternatives as it has been shown to have good biocompatibility and biodegradability, high bioavailability for praziquantel and offers sustained release of poorly water soluble drugs such as praziquantel and improved bioavailability of praziquantel in mice (Xie et al., 2010). Two types of nanoparticle were tested; regular HCO-SLN and the same particle coated with chitosan. Chitosan was expected to contribute to delayed drug release and thus improved palatability (Yuan et al., 2010). The improved mucoadhesion and transmucosal drug permeation attributed to chitosan may lead to improved drug absorption following oral administration.
Table 2.2 Composition, preparation technique and physiochemical properties of HCO-SLN and chitosan-coated SLN formulations

<table>
<thead>
<tr>
<th></th>
<th>HCO-SLN</th>
<th>HCO-10SLN-1CHI-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Praziquantel</td>
<td>100mg</td>
<td>100mg</td>
</tr>
<tr>
<td>HCO (solid lipid)</td>
<td>900mg</td>
<td>900mg</td>
</tr>
<tr>
<td>PVA (surfactant)</td>
<td>1% w/v, 20 mL</td>
<td>1% w/v, 20 mL</td>
</tr>
<tr>
<td>Chitosan</td>
<td>0mg</td>
<td>100mg</td>
</tr>
<tr>
<td>Preparation technique</td>
<td>HCO and drug was</td>
<td>HCO and drug was</td>
</tr>
<tr>
<td></td>
<td>emulsified in</td>
<td>emulsified in a</td>
</tr>
<tr>
<td></td>
<td>PVA solution,</td>
<td>premixed PVA and</td>
</tr>
<tr>
<td></td>
<td>homogenized, and</td>
<td>chitosan solution,</td>
</tr>
<tr>
<td></td>
<td>redispersed in 100</td>
<td>homogenized and</td>
</tr>
<tr>
<td></td>
<td>mL cold water.</td>
<td>redispersed in 100</td>
</tr>
<tr>
<td></td>
<td>The mixture was</td>
<td>mL cold water.</td>
</tr>
<tr>
<td></td>
<td>centrifuged at 12,000 rpm for 90</td>
<td>The mixture was</td>
</tr>
<tr>
<td></td>
<td>min at 4°C; the</td>
<td>centrifuged at 18,000 rpm (1h) and the</td>
</tr>
<tr>
<td></td>
<td>precipitate was</td>
<td>precipitate was lyophilized.</td>
</tr>
<tr>
<td></td>
<td>then redispersed</td>
<td></td>
</tr>
<tr>
<td></td>
<td>in 15 mL water</td>
<td></td>
</tr>
<tr>
<td>Drug loading %</td>
<td>7.87 ± 0.84%</td>
<td>7.99 ± 0.61%</td>
</tr>
<tr>
<td>Mean diameter</td>
<td>876.6 ± 114.6 nm</td>
<td>4651 ± 635 nm</td>
</tr>
<tr>
<td>Zeta potential</td>
<td>-12.2 ± 0.5 mV</td>
<td>+8.8 ± 0.7 mV</td>
</tr>
</tbody>
</table>

The gelatin used in this trial was sourced from Natures Grocer. Gelatin coated treatments were coated using a 20% (w/v) gelatin solution and the coating concentration was 50mL/kg of feed; this was then mixed thoroughly to ensure even coating for all gelatin treatments then the treatment was put in the fridge to set.

The transglutaminase used in this trial was sourced from Melbourne Food Ingredient Depot, Victoria Australia. It was prepared using 30 mg of transglutaminase per gram of protein (Sakamoto et al., 1994). The transglutaminase was added to a gelatin solution of 20% (w/v) (and the coating concentration was 50 mL/kg) this was then held at 50°C for 4 hours at a pH of 6. Following this the enzyme was deactivated by heating to 85°C for 20 minutes. This was achieved by preparing a bath in which a beaker sat. After 20
minutes the solution was cooled back to 50°C before being added to the 5 mm pellets pre-coated with praziquantel. The coating concentration of transglutaminase strengthened gelatin was also 50 mL/kg.

2.4 General methods

Trials were conducted in 20 200 L experimental tanks at the Australian Center for Applied Aquaculture Research (ACAAR) in Fremantle, Western Australia. Each tank was supplied with seawater with a flow rate of 2 L/min giving 14 exchanges a day. The incoming water created a circular flow path moving all waste towards a center standpipe to remove waste from the tanks. All tanks had a central air stone to maintain circulation and assist with waste removal. A header tank fed clean sea water to all the tanks. This header was oxygenated to ensure adequate and equal dissolved oxygen levels were maintained in all tanks. Each tank held 5 fish for the trial period to ensure a natural feeding response from the fish (i.e. yellowtail kingfish are a schooling fish and therefore require a certain school size to feed effectively) and to achieve statistically meaningful results. All fish were acclimated for 5 days once moved to the trial tanks before being switched to the trial diets. During this acclimation period the fish were fed non-medicated/uncoated control diet.

The fish received the treatment diets for a period of five days, this was based on recommended oral treatment methods (Stephens et al., 2003, Tubbs and Tingle, 2006b). The mean weight of fish and water temperature was used to determine the daily ration of feed (in percent body weight [body weight/kilogram]) based on Seriola feeding tables (Masumoto, 2002, Booth et al., 2010b). This ration was split in half and fed at
0900 and 1500 hours. The tables were used to ensure a full ration would be consumed and to prevent the ‘yo-yo’ or ‘ratchet’ feeding that can occur when fish are fed to satiety. Feed was given to each tank for a maximum of three minutes; at the end of the three minutes all remaining pellets were removed and counted. If all the feed was consumed within the three-minute period, the time taken to consume the diet was recorded.

Three different measures of palatability were calculated during the trials; percentage of feed consumed, percentage of times feed was finished (completely eaten) and time to finish feed. Percentage consumed was based on the amount of feed offered compared to the amount of feed remaining, averaged over the five-day period:

\[
\text{Percentage consumed} = \frac{\text{amount consumed}}{\text{amount offered}} \times 100
\]

Percentage of times finished data was calculated from tanks that completed the full ration within the 3-minute period.

\[
\text{Percentage of times finished} = \frac{\text{number of times finished within 3 minutes}}{\text{number of rations offered}} \times 100
\]

For those tanks which did finish the entire ration within three minutes the average time to consume the diet was also calculated.

Where possible, all three measures of palatability were used as response variables when comparing the effect of different treatments, although for trials where the feed was not finished, only percentage consumed could be used. Differences among treatments were tested by analyses of variance, followed by post hoc Tukey’s test, as
described for each trial (Section 2.5). All percentage data were normalized using arcsine transformation prior to analysis. All statistical analysis were undertaken using SPSS version 23.

### 2.5 Trial specific methods

Detailed methods of each trial are given in Table 2.3 below and described below.

**Table 2.3.** Treatments investigated and active praziquantel inclusion level for each treatment

<table>
<thead>
<tr>
<th>Trial</th>
<th>Treatment</th>
<th>Praziquantel incorporation</th>
<th>Active praziquantel inclusion level (g/kg food)</th>
<th>Coating</th>
<th>Number of replicates</th>
<th>Kingfish average weight (grams)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>Pure praziquantel in mash</td>
<td>8</td>
<td>0 mL/kg GB1</td>
<td>2</td>
<td>149 ± 25</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Pure praziquantel in mash</td>
<td>8</td>
<td>10 mL/kg GB1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Pure praziquantel in mash</td>
<td>8</td>
<td>20 mL/kg GB1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Pure praziquantel in mash</td>
<td>8</td>
<td>40 mL/kg GB1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Pure praziquantel on surface</td>
<td>8</td>
<td>10 mL/kg GB1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Pure praziquantel on surface</td>
<td>8</td>
<td>20 mL/kg GB1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Pure praziquantel on surface</td>
<td>8</td>
<td>40 mL/kg GB1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>HCO-SLN nanoparticle</td>
<td>5</td>
<td>gelatin coated</td>
<td>3</td>
<td>323 ± 5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>HCO-SLN nanoparticle</td>
<td>5</td>
<td>50 mL/kg GB1 direct fresh</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Pure praziquantel on surface</td>
<td>5</td>
<td>gelatin coated</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Pure praziquantel on surface</td>
<td>5</td>
<td>50 mL/kg GB1 direct fresh</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>None</td>
<td>0</td>
<td>gelatin coated</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>None</td>
<td>0</td>
<td>50 mL/kg GB1 direct fresh</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>None</td>
<td>0</td>
<td>None</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>Pure praziquantel on surface</td>
<td>5</td>
<td>50 mL/kg GB1 old</td>
<td>4</td>
<td>328 ± 5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Pure praziquantel on surface</td>
<td>5</td>
<td>50 mL/kg GB1 fresh</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>HCO-CHI nanoparticle</td>
<td>5</td>
<td>gelatin coated</td>
<td>3</td>
<td>332 ± 5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>HCO-CHI nanoparticle</td>
<td>5</td>
<td>50 mL/kg GB1 direct fresh</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Pure praziquantel on surface</td>
<td>5</td>
<td>gelatin coated</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Pure praziquantel on surface</td>
<td>5</td>
<td>50 mL/kg GB1 direct fresh</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>None</td>
<td>0</td>
<td>gelatin coated</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>None</td>
<td>0</td>
<td>50 mL/kg GB1 direct fresh</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>None</td>
<td>0</td>
<td>Control</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>Pure praziquantel on surface</td>
<td>5</td>
<td>Transglutaminase gelatin coated</td>
<td>4</td>
<td>336 ± 5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Pure praziquantel on surface</td>
<td>5</td>
<td>gelatin coated</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>Pure praziquantel on surface</td>
<td>5</td>
<td>25 mL/kg GB1</td>
<td>3</td>
<td>347 ± 5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Pure praziquantel on surface</td>
<td>10</td>
<td>25 mL/kg GB1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Pure praziquantel on surface</td>
<td>15</td>
<td>25 mL/kg GB1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Pure praziquantel on surface</td>
<td>5</td>
<td>50 mL/kg GB1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Pure praziquantel on surface</td>
<td>10</td>
<td>50 mL/kg GB1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Pure praziquantel on surface</td>
<td>15</td>
<td>50 mL/kg GB1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>none</td>
<td>0</td>
<td>none</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>
2.5.1 Trial 1. Compare application methods and garlic extract concentration on the palatability of diets containing praziquantel

Trial 1 compared two application methods of praziquantel and three GB1 concentrations on the palatability of diets containing praziquantel. Praziquantel was either applied to the surface of the diet with one of three GB1 concentrations, or it was incorporated into the pellet mash prior to extrusion into pellets then top coated with the three same concentrations of GB1 as shown in Table 2.3. A treatment of mash diet to which no GB1 was applied was included however a surface coated treatment without GB1 could not be included because the praziquantel by itself would not adhere to the surface of the pellets. The diets used in this trial were prepared from a commercial yellowtail kingfish mash containing 45% protein and 20% lipid (Ridley Agriproducts; www.ridley.com.au) and extruded into 4 mm diameter pellets using a Wenger X-85 extruder at the Australasian Experimental Stockfeed Extrusion Centre, South Australia. Prior to extrusion, pure praziquantel was added to one portion of the mash at the rate of 8 g/kg. The remaining portion was extruded into pellets without praziquantel, then surface coated with pure praziquantel to form the various surface coated treatment diets outlined in Table 2.3. Pure praziquantel treatments that were surface coated were mixed thoroughly until even coating was achieved. Following this, GB1 was added and again thoroughly mixed to ensure even coating over all pellets. All treatments were stored in a cool room for 5 days prior to treatment feeds starting. The mean fish weight over all treatments was 149 ± 25g and there was no significant difference in fish weight between tanks.
The effects of the method of praziquantel application (surface or in mash) and concentration of GB1 (10, 20 or 40 mL/kg) on percentage consumed data were analysed by two-way ANOVA. For the mash diets, only the effect of concentration of GB1 (0, 10, 20 or 40 mL/kg) were compared in a separate one-way ANOVA.

2.5.2 Trial 2. Determine whether incorporating praziquantel into hydrogenated castor oil solid-lipid nanoparticles (HCO-SLN) improves palatability of diets containing praziquantel

Trial 2 tested whether incorporating praziquantel into hydrogenated castor oil solid-lipid nanoparticles (HCO-SLN) improved palatability of diets containing praziquantel. Seven different treatments were use; HCO-SLN nanoparticle with GB1 coating; HCO-SLN nanoparticle with gelatin coating; pure praziquantel with GB1 coating; pure praziquantel with gelatin coating; GB1 coating without praziquantel; gelatin coating without praziquantel; and feed with no coating or praziquantel. The praziquantel inclusion level for all the treatments which contained the drug was 5 g/kg. There were three replicates for all the coating treatments and two replicates for the control with no coating.

The feed used in this trial was Skretting 5mm Nova FF pellets. Fifty mL/kg of GB1 was used for each of the GB1 treatments and was applied to the pellets 30 mins before being fed; this contrasts with trial 1, where the GB1 coating was prepared 5 days prior to the trial. Freshly prepared coating was used in this trial due to concerns about nanoparticles
leaching praziquantel into the GB1 and therefore reducing palatability. The gelatin coated treatments were coated using a 20% (w/v) gelatin solution also at 50mL/kg and stored in a cool room to set. The mean fish weight for this trial was 323 ± 5g with no significant difference in fish weight between tanks.

The effects of the method of praziquantel application (surface or in nanoparticles) and method of coating (gelatin or GB1) on percentage consumed and percentage times finished were analysed by two-way ANOVA. Time taken to finish data was analysed by two separate one-way ANOVA, firstly to compare praziquantel application method and secondly the method of coating. Diets without praziquantel were excluded from this analysis and were used to confirm that neither coating method affected palatability.

2.5.3 Trial 3. Test the hypothesis that freshly applying garlic extract to diets containing praziquantel improves palatability relative to those coated 5 days prior to use

Trial 3 was undertaken because of the results from Trials 1 and 2, and tested whether freshly applying GB1 to diets containing praziquantel improves palatability, relative to those coated 5 days prior to use. There were 2 different treatments in this trial GB1 ‘fresh’ and GB1 ‘old’. The praziquantel inclusion level for all the treatments was 5 g/kg. There were 4 replicates for each treatment.

The feed used in this trial was Skretting 5mm Nova FF pellets. Fifty mL/kg of GB1 was used for each of the GB1 treatments and was applied to the pellets 30 mins before being fed for the GB1 fresh treatment. The GB1 old treatment was made five days prior to
feeding and was stored in a cool room after being coated. The mean fish weight for this trial was 328 ± 5g with no significant difference in fish weight between tanks.

Percentage consumed, percentage times finished and time taken to finish were compared among treatments by one-way ANOVA.

2.5.4 Trial 4. Determine whether incorporating praziquantel into chitosan coated HCO-SLN improves palatability of diets containing praziquantel

This trial followed the same methods as described for Trial 2, except that HCO nanoparticles coated in chitosan were used. The mean fish weight for this trial was 332 ± 5g with no significant difference in fish weight between tanks.

The effects of the method of praziquantel application (surface or chitosan-coated nanoparticles) and method of coating (gelatin or GB1) on percentage consumed, percentage times finished and time taken to finish were analysed by two-way ANOVA. Diets without praziquantel were excluded from this analysis and were used to confirm that neither coating method affected palatability.

2.5.5 Trial 5. Determine whether strengthening the gelatin binder improves palatability of diets containing praziquantel

This trial contained two treatments; one with normal gelatin and one with gelatin strengthened with transglutaminase. The praziquantel inclusion level was 5g/kg of feed, there was four replicates for each treatment.

The feed used in this trial was Skretting 5mm Nova FF pellets. The transglutaminase was prepared using the methods described above. The gelatin coated treatments were
coated using a 20% (w/v) gelatin solution and stored in a cool room to set. The mean fish weight for this trial was 336 ± 5g with no significant difference in fish weight between tanks.

Percentage consumed, percentage times finished and time taken to finish were compared among treatments by one-way ANOVA.

2.5.6 Trial 6. Determine the interactive effect of praziquantel inclusion level and garlic extract concentration on palatability of diets containing praziquantel

Trial 6 tested the interactive effect of praziquantel inclusion level and GB1 concentration on palatability of diets containing praziquantel. This trial had seven different treatments; six of these treatments differed in their inclusion level of praziquantel (5, 10 or 15g/kg of feed) and their concentration of GB1 coating (25 or 50 mL/kg of feed), while the final treatment was a control with no praziquantel and no coating. There were three replicates for all the coating treatments and two replicates for the control.

The feed used in this trial was Skretting 5mm Nova FF pellets. All treatments were prepared fresh 30 minutes before being fed to the fish. The mean fish weight for this trial was 347 ± 5g with no significant difference in fish weight between tanks.

The effects of praziquantel inclusion level and GB1 concentration on percentage consumed, percentage times finished and time taken to finish were analysed by two-way ANOVA. Diets without praziquantel were excluded from this analysis, but were used for comparative purposes.
3 Results

3.1 Trial 1

The effect of different praziquantel application methods (incorporated in mash or surface coated) and different GB1 concentrations on the percentage of ration eaten is shown in Figure 3.4. Due to availability of tanks, no control treatments (diets without praziquantel) were included in this study, however all fish in tanks ate 100% of their control ration during the acclimation period. Two-way ANOVA revealed a significant effect of application method ($p = 0.02$), but no significant effect of GB1 concentration ($p = 0.09$) or interaction (0.17). Post hoc tests showed that fish ate significantly less of the mash diets (least square mean = 35%) than those which were surface coated (60%).

Figure 3.2 shows the effect of GB1 concentration (including 0 GB1) on the percentage of ration consumed in the mash diets. One way ANOVA found a significant effect of GB1 concentration ($p = 0.01$), with fish fed the diet coated with 40 mL/kg of GB1 eating significantly more food (62%) compared to those offered the diets with no GB1 (25%), 10 mL/kg (25%) and 20 mL/kg (19%).

For this study time data was not recorded.
Figure 3.1 Effect of method of application of praziquantel (surface of feed or incorporated into the mash) and GB1 concentration on the percentage of feed consumed by yellowtail kingfish. Treatment combinations with different letters differed significantly (p < 0.05).

Figure 3.2 Effect of GB1 concentrations on the percentage of feed consumed by yellowtail kingfish. Treatments with different letters differed significantly (p < 0.05).
3.2 Trial 2

The effects of the method of praziquantel application (surface or in nanoparticles) and method of coating (gelatin or GB1) on the various response variables are shown in Figures 3.3 to 3.5. All three diets containing no praziquantel were fully consumed 100% of the time, in $16 \pm 3$ seconds, demonstrating no negative impact of gelatin coating or GB1 coating on palatability.

There was a significant effect of method of coating on percentage of the ration consumed (two-way ANOVA; $p = <0.001$) and the percentage of times the ration was finished ($p = 0.001$). Post hoc tests showed that fish offered GB1 coated diets consumed significantly more of these diets (least squared mean = 78%) and consumed the entire ration on significantly more occasions (56%) than those coated with gelatin (49% and 7%, respectively).

There was a significant effect of praziquantel form on percentage of the ration consumed ($p = <0.001$) and percentage of times a full ration was consumed ($p = 0.001$). Post hoc tests showed that fish ate significantly less of the diets coated with HCO-SLN nanoparticles (49%), consumed the entire ration significantly fewer times (8%) and took significantly longer to consume the ration (150 seconds) than those coated with pure praziquantel (78%, 55% and 50 seconds, respectively).

There was a significant effect of the interaction between application method and method of coating on the percentage consumed ($p = 0.002$) and percentage of times completed ($p = 0.003$). This interaction arose because the difference between application methods was greater in diets coated with GB1 than with gelatin (Figs 3.3, 3.4).
Time to consume a complete ration (Figure 3.5) data was analysed with two separate one-way ANOVA’s. There was a significant effect of praziquantel form in the GB1 coated diets (p=<0.001). There was no significant effect of application method in the diets containing pure praziquantel (p=0.06) although the gelatin coated diets took longer to finish (84 seconds) compared to the GB1 coated diets (38 seconds).

Figure 3.3 Effect of method of application of praziquantel (surface of feed or incorporated into nanoparticle) and method of coating (gelatin or GB1) on the percentage of feed consumed by yellowtail kingfish.
Figure 3.4 Effect of method of application of praziquantel (surface of feed or incorporated into nanoparticle) and method of coating (gelatin or GB1) on the percentage of times total ration consumed by yellowtail kingfish.

Figure 3.5 Effect of method of application of praziquantel (surface of feed or incorporated into nanoparticle) and method of coating (gelatin or GB1) on the average time for total ration consumed by yellowtail kingfish.

3.3 Trial 3
The effect of freshly coated GB1 (fresh) against GB1 that had been coated and stored for 5 days (old) on palatability is shown in Figures 3.6-3.8. Freshly coated GB1 ration
was preferred when measured by percentage consumed (one-way ANOVA; $p = 0.03$; 
(97 ± 3% compared to 76 ± 10% for ‘old’ GB1), and percentage of times finished ($p = 
0.008$; 93 ± 7% compared to 38 ± 14%), although the difference between the two 
treatments time to be consumed was not quite significant ($p = 0.06$; 22 seconds 
compared to 67 seconds).

Figure 3.6 Effect of freshly coated GB1 (fresh) against GB1 that had been coated and 
stored for 5 days (old) on the percentage of feed consumed by yellowtail kingfish. 
Treatments with different letters differed significantly ($p < 0.05$).
Figure 3.7 Effect of freshly coated GB1 (fresh) against GB1 that had been coated and stored for 5 days (old) on the percentage of time total ration was consumed by yellowtail kingfish. Treatments with different letters differed significantly (p < 0.05).

![Chart showing the effect on consumption time.]

Figure 3.8 Effect of freshly coated GB1 (fresh) against GB1 that had been coated and stored for 5 days (old) on the average time for the total ration to be consumed by yellowtail kingfish. Treatments with different letters differed significantly (p < 0.05).

3.4 Trial 4
The effect of method of praziquantel application (surface or in HCO nanoparticles coated with chitosan) and method of coating (gelatin or GB1) on the various response variables are shown in Figures 3.9 to 3.11. All three diets containing no praziquantel consumed 100% their diets 100% of the time and completed their diet in 18 ± 3 seconds, demonstrating no negative impact of gelatin coating or GB1 coating.

There was a significant effect of coating method on percentage of the ration eaten (two-way ANOVA; p = <0.001) and the percentage of times a full ration was eaten (p = <0.001) but no significant effect on time taken to complete ration (p = 0.80). Post hoc tests showed that fish consumed significantly more GB1 coated diets (least squared
mean = 89%) and consumed the entire ration on significantly more occasions (71%) than gelatin coated diets (56% and 35%, respectively).

There was a significant effect of method of praziquantel application percentage consumed \((p < 0.001)\), percentage times consumed \((p < 0.001)\) and time taken to complete ration \((p = 0.003)\). Post hoc tests showed that fish ate significantly less of the diets coated with HCO-SLN nanoparticles (51%), finished these diets significantly fewer times (10%) and took significantly longer to finish (157 seconds) than those coated with pure praziquantel on the surface (66%, 50% and 36 seconds, respectively.

There was a significant effect of the interaction between application and coating methods on the percentage consumed \((p = 0.002)\) and percentage completed \((p = 0.003)\), but not on the time to finish \((p = 0.37)\). This interaction arose because the difference between application methods was greater in diets coated with GB1 than with gelatin (Figs 3.9, 3.10).
Figure 3.9 Effect of method of application of praziquantel (surface of feed or incorporated into nanoparticle coated with chitosan) and method of coating (gelatin or GB1) on the percentage of feed consumed by yellowtail kingfish.

Figure 3.10 Effect of method of application of praziquantel (surface of feed or incorporated into nanoparticle coated with chitosan) and method of coating (gelatin or GB1) on the percentage of times total ration was consumed by yellowtail kingfish.

Figure 3.11 Effect of method of application of praziquantel (surface of feed or incorporated into nanoparticle coated with chitosan) and method of coating (gelatin or GB1) on the average time for a full ration to be consumed by yellowtail kingfish.
3.5 Trial 5

The effect of coating feed with gelatin strengthened with transglutaminase compared to regular gelatin coating on palatability is shown in Figures 3.12-3.1. There was no significant effect of preparing treatment with strengthened gelatin on either the percentage of ration consumed (one-way ANOVA; $p = 0.84$), the percentage of times the ration was finished ($p = 0.88$) or on the time to be consumed ($p = 0.50$).

Figure 3.12 The effect of coating feed with gelatin strengthened with transglutaminase compared to regular gelatin coating on the percentage of feed consumed by yellowtail kingfish.
Figure 3.13 The effect of coating feed with gelatin strengthened with transglutaminase compared to regular gelatin coating on the percentage of times a total ration was consumed by yellowtail kingfish.

Figure 3.14 The effect of coating feed with gelatin strengthened with transglutaminase compared to regular gelatin coating on the average time taken for a total ration to be consumed by yellowtail kingfish.
3.6 Trial 6
The effect of the interaction of different concentrations of praziquantel and GB1 on the various response variables are shown in Figures 3.15 to 3.17. Despite a general trend of a decline in all response parameters with increasing praziquantel dietary inclusion levels, there was no significant effect of praziquantel dietary inclusion level on percentage of the ration eaten (two-way ANOVA; $p = 0.19$), the percentage of times a full ration was eaten ($p = 0.22$) or on time taken to complete the ration ($p = 0.62$).

Similarly, there was no significant effect of GB1 concentration on percentage of ration consumed ($p = 0.34$) or percentage of times consumed ($p = 0.35$) or time taken to complete ration ($p = 0.051$). There was a trend for diets coated with 25mL/kg of GB1 to be consumed more slowly (69 seconds) than diets coated with 50mL/kg of GB1 (39 seconds), but this was not quite significant ($p = 0.05$).

There was no significant effect of the interaction between praziquantel inclusion level and GB1 concentration on either the percentage consumed ($p = 0.70$), percentage of times completed ($p = 0.82$), or time to finish ($p = 0.51$).

Diets containing no praziquantel and no GB1 consumed 100% of their diets 100% of the time and completed their diet in 16 ± 2 seconds.
Figure 3.15 The effect of the interaction of different concentrations of praziquantel and GB1 on the percentage of feed consumed by yellowtail kingfish.

Figure 3.16 The effect of the interaction of different concentrations of praziquantel and GB1 on the percentage of times a full ration was consumed by yellowtail kingfish.
Figure 3.17 The effect of the interaction of different concentrations of praziquantel and GB1 on the average time for a full ration to be consumed by yellowtail kingfish.

4 Discussion

4.1 Efficacy of flavour-masking techniques

One of the most concerning diseases of cultured yellowtail kingfish is parasites (Sánchez-García et al., 2014). Chemical treatment is the principal method used to control parasites in the aquaculture industry. The most commonly used chemicals to treat infections are sodium chloride (in freshwater fish), formaldehyde, copper sulfate, hydrogen peroxide and anthelmintic drugs such as mebendazole, trichlorphon and praziquantel (Eiras et al., 2008). These therapies have two major administration methods, being bathing in the chemicals or oral medication. Oral administration of medications to sea cage fish has advantages over conventional bath treatments. Overall, the inclusion of oral treatments requires less labour and time, as well as less stress for the fish (Grant, 2002).
Praziquantel’s strong bitterness has resulted in appetite suppression and diet rejection in yellowtail kingfish (Partridge et al., 2014). Methods to achieve taste masking include applying medication directly into feed during processing, coating with flavours, binders and encapsulation. (Oppel, 2008). This study hypothesised that the palatability to yellowtail kingfish of feed containing praziquantel could be improved through innovative methods such as incorporating the praziquantel into the mash prior to extrusion, flavour masking, the use of stronger binders and incorporation of praziquantel into nanoparticles. The results revealed that flavour masking with garlic extract (GB1) was successful at improving the palatability of feed containing praziquantel. The use of mash diets, stronger binders and nanoparticles, however, proved ineffective at increasing the palatability of praziquantel diets. These findings have considerable implications for improving the efficiency and limiting the productivity side-effects of the treatment of yellowtail kingfish.

4.1.1 Garlic extract

The application method that proved most effective was surface coating praziquantel with GB1. Inclusion of praziquantel into the mash followed by GB1 coating was the least effective method in Trial 1. Palatability of the mash diets were only improved with the addition of GB1 at the highest concentration tested (40 mL/kg). Trial 3 tested the effect of coating GB1 ‘fresh’ against GB1 that had been coated and stored for 5 days (‘old’), which was the method employed in Trials 2 and 4. This trial found that ‘fresh’ GB1 diets were consumed significantly better than ‘old’ GB1 diets. It appears that the extract and praziquantel interact when they are in contact with each other for an
extended period (5 days stored). This does not seem to be the case when GB1 is applied ‘fresh’. This trial provided vital data for further research in this study. Trial 6 showed that when GB1 is applied ‘fresh’ there is no significant effect of concentration of GB1 (25 or 50 mL/kg) on the palatability of the diet. This result also showed that up to 15 g/kg of praziquantel could be ingested in high amounts with both GB1 concentrations. This appears to be the first study demonstrating the use of garlic extract as a flavour masking agent and is also one of the first studies demonstrating any effective masking of praziquantel’s bitter flavor. Partridge et al (2012), for example, tested five flavor masking agents and none were as effective as the garlic extract trialed here.

The use of this garlic extract may have other positive impacts on fish health. It has been shown that garlic has antibacterial and antifungal properties (Guo et al., 2012) as well as anthelmintic properties (Pena et al., 1988). The combination of garlic extract and praziquantel may therefore have a dual positive impact of fluke eradication and prophylactic health benefits. Further research is required on the effect of garlic extract on fish health and production along with the possible positive interaction as a masking agent for praziquantel.

4.1.2 Mash diets

The incorporation of praziquantel into mash in Trial 1 proved ineffective at increasing palatability of the diet. This disproved my hypothesis that inclusion of praziquantel into the mash before extrusion would increase palatability relative to surface coating. This result contrasts to previous studies with antibiotics where mash diets proved more
effective than surface coating feeds (Rigos et al., 1999). In a previous study, Partridge et al (2014) also found no clear benefit of incorporating praziquantel into mash prior to extrusion. Despite the findings of Partridge et al (2014) showing no benefit of mash inclusion, this trial was conducted to determine if GB1 coating these mash diets would make them more palatable relative to surface-coated diets. The negative result from my trial may be because the mash feed used was the same as that used in Partridge et al (2014), and the praziquantel may have interacted with the feed. The age of the diet itself was not the cause, as the acclimation feed (without praziquantel) was from the same batch of feed and was well ingested. This suggests that the praziquantel may have leached throughout the pellets causing poor flavour.

While the age of these praziquantel incorporated, diets may have contributed to their poor performance, it was decided not the investigate mash diets further in this study because this method would be expensive and difficult to apply on a commercial scale. This is because feed would have to be prepared with different praziquantel dietary inclusion levels for different sized fish (Table 4.1), meaning the feed would have to be made to order (Williams et al., 2007). This approach would also require feed companies to shut down and clean machinery after each medicated batch to ensure residual praziquantel did not contaminate non-medicated feed, as medications have withholding periods on fish that will be supplied for human consumption (Tubbs and Tingle, 2006a).

4.1.3 Increasing binder strength
This study found that were no significant differences between transglutaminase strengthened gelatin and regular gelatin in improving the palatability of feed containing praziquantel. This disproved the hypothesis that the inclusion of transglutaminase to the gelatin coating would increase binding strength and withhold the bitterness of praziquantel from the fish. Transglutaminase was selected as possible solution to increase the palatability of praziquantel surface coated feeds as it has been shown to greatly increase the binding strength of gelatin (Sakamoto et al., 1994, Fuchs et al., 2010). In a previous study, Partridge et al (2012) tested a range of different binder concentrations of gelatin, and found some positive results from the use of gelatin as a surface coating. The increased strength of the gelatin by adding transglutaminase appeared to be insufficient in masking the bitter flavour of praziquantel at the concentrations used in this trial.

4.1.4 Nanoparticles

This study also found no evidence that praziquantel incorporated into nanoparticles had improved palatability compared to pure, unincorporated praziquanl. It had been hypothesised that palatability would be improved because encapsulated praziquantel is not in direct contact with the fish’s taste buds. The failure to improve palatability may be attributed to the volume of nanoparticle that needed to be used to achieve the desired active praziquantel inclusion level. The high volume of nanoparticles required was due to the low loading rates of praziquantel in the nanoparticles (10% loading rate). The nanoparticle diets therefore had a much greater volume of product coating the diet than the pure praziquantel diets. The increased powder coating may have made the diet not
smell and/or taste like normal fish feed, thereby reducing its palatability. Future studies may be able to test whether the volume was affecting the palatability of the feed by creating nanoparticles without praziquantel and just testing the effect of volume on palatability.

Nanoparticles were tested due to results of previous studies with microencapsulated praziquantel that had an increased palatability of praziquantel (Partridge et al., 2014), but reduced bioavailability of the praziquantel making it less effective as a treatment. The microcapsules had an active inclusion level of 40% whereas the nanoparticle had an active inclusion level of 10%. The benefits of nanoparticles are that they have been shown to provide a greater bioavailability of praziquantel in mice (Xie et al., 2010). However, a recent study found no increase in the bioavailability of praziquantel using the same nanoparticles in (Partridge et al., 2016). This, combined with evidence from my study that nanoparticles had a negative effect on palatability suggests that further research on using this technique to incorporate praziquantel in kingfish feed is not warranted.

4.2 Inclusion level of praziquantel for treatment of flukes

The most important parasites of yellowtail kingfish in Australia are the monogenean flukes, *Z. seriolae* and *B. seriolae* (Sharp et al., 2004). The recommended dose of praziquantel for treating skin and gill flukes is 50-150 mg/kg of body weight per day for 3-5 days (Stephens et al., 2003, Tubbs and Tingle, 2006b). This range in recommended doses is in relation to the type of parasite that is being treated. *Zeuxapta seriolae* (gill
fluke) can be treated with the lower dose of 50 mg/kg/day as these parasites feed directly on blood where praziquantel concentration is relatively high (Mooney et al., 2006). *Benedenia seriolae* (skin fluke) feeds on the mucus and epithelial cells of the fish (Chambers and Ernst, 2005), meaning a much higher dose of praziquantel (150 mg/kg/day) is required and for a longer period to treat this species to allow for the praziquantel to be transferred from the blood into the mucus and epithelial cells of the fish.

The relationship between the dietary inclusion level of praziquantel in the diet and the dose received by the fish is affected by the size of the fish and the temperature of the water. These two variables are used to determine the amount of feed the fish should eat. This means larger fish require a higher dietary inclusion level of praziquantel to achieve the same dosage. For example, treating a 50 g fish at 24 °C for gill flukes requires only 0.8 g of praziquantel per kilogram of diet whilst treating a 3 kg fish at 15 °C for skin flukes requires 19 g/kg of feed. An example of how the relationship of fish size and water temperature can affect the dietary inclusion level of praziquantel in feed is shown in Table 4.1. This table demonstrates the challenging issues that occur as fish get larger and temperature decreases. The increased dietary inclusion level in larger fish combined with the increased sensitivity of taste in larger fish may reduce the palatability of these diets.

This study tested a range of praziquantel dietary inclusion levels to determine if different concentrations could successfully be used. The varying inclusion levels tested in Trial 6 were 5, 10 and 15 g/kg. These inclusion levels were equivalent to 53, 102 and 122 mg/kg/day respectively in dosage rates based on the amount of food these fish ate.
From these results these fish should have had the required dose over the five-day period to treat both skin and gill flukes.

Table 4.1 The relationship between fish weight, % body weight of feed recommended (Matsumoto, 2002), the calculated amount of feed one fish would consume at required bodyweight percentage for different temperatures, required praziquantel dietary inclusion level in g/kg for 50 and 150mg/kg of bodyweight doses to treat different fluke species.

<table>
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<th>Fish weight (grams)</th>
<th>Ration (% BW/day)</th>
<th>Praziquantel dietary inclusion level (g/kg) to achieve 50mg/kg</th>
<th>Praziquantel dietary inclusion (g/kg) to achieve 150mg/kg</th>
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This study has uncovered a possible solution for orally medicating commercially cultured yellowtail kingfish with praziquantel. Further studies will be required for example; this study was not able to find the absolute minimum GB1 dose needed for palatability. The first trial tested a lower GB1 dose of 10 mL/kg but this trial was compromised by the fact that the garlic extract was applied 5 days prior to use. Further studies testing lower concentrations would be beneficial as reduced GB1 concentration would mean reduced costs for the aquaculture industry. Alongside this, the maximum praziquantel dietary inclusion level was not found. This will be important for treating fish of different weights and at different water temperatures. As fish get larger it is believed that their taste sensitivity may increase compared to that of smaller fish. Due to this, a similar experiment should be undertaken with larger fish to test if they will consume diets with high praziquantel inclusion levels necessary to administer the higher doses required to treat skin flukes. Finally, to test the efficacy of surface coating of
praziquantel, a trial will need to be conducted on fish that are infected with flukes to confirm its effectiveness under this form of application. This is important as surface coating may appear to be being consumed but may be washed off the feed before the fish can ingest the medication. This result may also find if fish eat 80% of a diet that the treatment is still effective.

5 Conclusion

Investigations into several innovative approaches to improve the palatability of feed containing praziquantel for commercially cultured yellowtail kingfish revealed garlic extract applied ‘fresh’ to the surface of pellets coated with praziquantel increased the palatability of this feed. These medicated diets approached 100% of total consumption. There was evidence of reduced palatability as praziquantel inclusion levels increased and garlic extract levels decreased. It was found that incorporation of praziquantel into mash feeds before pellet extrusion, incorporation of praziquantel into hydrogenated castor oil solid lipid nanoparticles and the use of transglutaminase to strengthen a binder such as gelatin proved ineffective at increasing the palatability of these feeds. This study focused on juvenile kingfish no bigger than 350grams. Future research should revolve around larger kingfish, reducing the concentration of GB1 required and testing efficacy in fish infested with flukes. The research conducted in this study may have significant implications for the future treatment of finfish in the aquaculture industry.
References


Appendix 1.

From Matsumoto 2002

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%BW daily intake VS temperature

Yellow areas are extrapolated