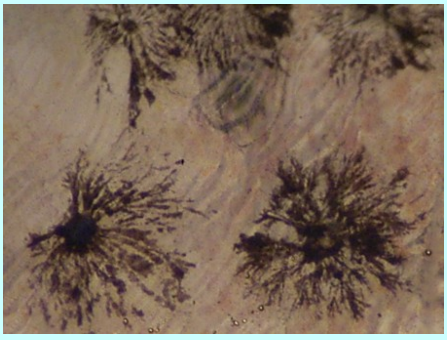
Final Report

AQUAFIN CRC PROJECT NO. 1B.3: INCREASING THE PROFITABILITY OF SNAPPER FARMING BY IMPROVING HATCHERY PRACTICES AND DIETS
VOLUME 2: SKIN COLOUR

Ben J. Doolan, Mark A. Booth, Geoff L. Allan and Paul L. Jones

September 2008

FRDC Project No. 2001/208



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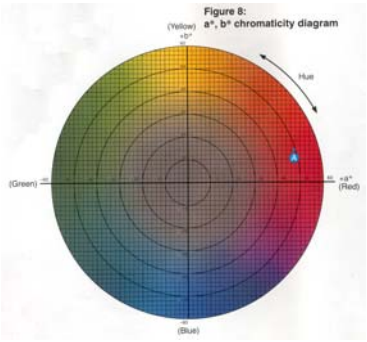
LIST OF ACRONYMS

*a**	Skin redness
$AUD	Australian dollar
ABARE	Australian Bureau of Agricultural & Resource Economics
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
*b**	Skin yellowness
BASF	Badische Anilin und Soda Fabrik (German chemical products company)
BW	Body weight
CIE	Commission Internationale de l'Eclairage
CRC	Cooperative Research Centre
CSIRO	Commonwealth Scientific & Industrial Research Organisation
DPI	Department of Primary Industries
FBW	Final body weight
FRDC	Fisheries Research & Development Corporation
IBW	Initial body weight
K^+	Potassium
KCl	Potassium chloride
*L**	Skin lightness
Mmol	Milli mole
NATA	National Association of Testing Authorities
NCFC	Newcastle Commercial Fisherman's Cooperative
PAR	Photosynthetically active radiation
PSFC	Port Stephens Fisheries Centre
PVC	Polyvinyl chloride
RAS	Recirculating aquaculture system
SBA	Silver Beach Aquaculture
SD	Standard deviation
SFM	Sydney Fish Market
SEM	Standard error of mean
SGR	Specific growth rate
SNK	Student Newmans Kuels test



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EXECUTIVE SUMMARY

Australian snapper (*Pagrus auratus)*, known as red sea bream in Japan, is a premium table fish that fetches high market prices in eastern Australia. Commercial culture of this species has been constrained by the high costs of feeds and feeding and fingerlings. The work described in this report (in three volumes) details results of research to address these constraints. This research has increased our knowledge of the nutritional requirements of Australian snapper *Pagrus auratus* and provided information on the potential of Australian feed ingredients to reduce the level of fishmeal in diets for this species. To meet the general and specific aims of this study, a research strategy based on determination of apparent digestibility coefficients to evaluate the potential for a range of potential feed ingredients, establishment of requirements for digestible energy and protein, and an assessment of how well different nutrients from key ingredients was adopted. Nutritional requirements are presented here as are details of the value of different feed ingredients. Trials were conducted that showed that fishmeal could be reduced to approximately 16% provided digestible energy and protein contents were maintained.

Farmed snapper are generally darker and less red in appearance than those obtained from the wild harvest, leading to lower market prices. This issue was raised as a priority among pioneer snapper farmers in Australia. The research described here has increased our knowledge of the factors that affect skin colour and developed practical methods to improve the appearance of farmed snapper. The combination of culturing snapper in light coloured tanks and feeding diets high in the natural pigment, astaxanthin, produced 'pink" coloured snapper that met consumer preferences.

Before this project started, snapper larvae were cultured in hatcheries using intensive techniques and while several hatcheries had successfully produced fingerlings, production costs were high. During this project, new protocols for intensive production were developed for managing environmental variables such as light intensity, photoperiod and temperature and live and inert feeds and feeding regimes. These new protocols allowed greatly increased hatchery production and lower costs. In addition, prior to this project there was no information on the potential or procedures to culture larvae extensively in outdoor, fertilised ponds. Results presented here have provided an understanding of zooplankton production in ponds and allowed strategic stocking of snapper larvae. High quality juvenile snapper were successfully produced in large numbers in fertilised ponds, demonstrating that extensive larval rearing of snapper is a viable alternative to traditional intensive culture.

Snapper, in common with other marine fish, occasionally suffer from infestation of the ectoparasite, *Amyloodinium ocellatum.* During this project, this parasite was genetically characterised and an understanding was made of the methods in which infestation can take place within hatcheries. A variety of control methods including chemotherapeutics were evaluated and recommendations for hatchery biosecurity were made.

This research has been transferred to industry through publications and workshops. Three PhD projects were completed during the project, two have been awarded and the last one will be submitted soon.

Although snapper was the focus of early marine finfish farming operations in New South Wales, Western Australia, South Australia and Queensland, more recently, emphasis has shifted to other marine species, mulloway (*Argyrosomus japonicus*) and yellowtail kingfish (*Seriola lalandi*). Much of the research described in this series of reports is relevant to all temperate species of marine fish and research methods used are generally applicable for a range of aquaculture species facing similar constraints.

NON-TECHNICAL SUMMARY

2001/208	Aquafin CRC – Increasing the profitability of snapper farming by improving hatchery practices and diets

PRINCIPAL INVESTIGATOR: Dr Geoff L. Allan

ADDRESS: NSW Department of Primary Industries
Port Stephens Fisheries Centre
Private Bag 1
Nelson Bay, NSW, 2315
Telephone: 02 4982 1232 Fax: 02 4982 1107
Email: Geoff.Allan@dpi.nsw.gov.au

OBJECTIVES:

(1) Improve production of snapper fingerlings by developing extensive, fertilised-pond rearing techniques for the advanced production of snapper juveniles.

(2) Improve production of snapper fingerlings by developing larval feeding strategies to reduce the use of live feeds, in particular *Artemia*, by weaning larvae at an early age onto commercial and/or experimental artificial diets.

(3) Improve production of snapper fingerlings by developing methods to reduce and/or treat the incidence of parasite infestation.

(4) Improve the skin colour of farmed snapper by reducing melanisation and improving skin pigmentation.

(5) Determine digestibility for, and ability of fish to utilize, new ingredients with potential for use in low-polluting snapper diets.

(6) Evaluate ability of snapper to utilize carbohydrate and lipid sources for energy.

(7) Determine optimum protein:energy ratio for fish grown at one favourable temperature.

(8) Provide recommendations for feeding strategies to minimise overfeeding and maximise fish production.

This final report is published in three volumes. Objectives 5, 6 and 7 pertain to Volume 1: Diet Development; **Objective 4 pertains to this Volume 2: Skin Colour**; Objectives 1, 2, 3 and 8 pertain to Volume 3: Fingerling Production and Health.

The Executive Summary, Background, Need, Objectives, Benefits and Adoption, Further Development and Planned Outcomes are common to all three volumes.

NON TECHNICAL SUMMARY:

OUTCOMES ACHIEVED

Australian snapper *Pagrus auratus*, known as red sea bream in Japan, is a premium table fish that fetches high market prices in eastern Australia. Farmed snapper are generally darker and less red in appearance than those obtained from the wild harvest, leading to lower market prices. This issue was raised as a priority among pioneer snapper farmers in Australia. The research described in this Volume has increased our knowledge of the factors that affect skin colour and developed practical methods to improve the appearance of farmed snapper. Throughout the research, the skin colour of snapper was measured using a handheld chromameter and colour was quantified according to the L^* (lightness), a^* (green to red), b^* (blue to yellow) colour space.

Two primary mechanisms determine skin colour in snapper. The first involves production and control of melanin, a black pigment, in melanophores in the skin. Melanin production and expression in the skin can change in response to environmental conditions. The second response involves the deposition and control of the red carotenoid pigment, astaxanthin, in erythrophores in the skin. Astaxanthin in the skin is related to the intake of feeds containing astaxanthin (in the wild this would include natural prey items such as prawns or other small crustaceans) and in cultured fish is supplied via the formulated diet. A systematic approach was taken to investigate factors controlling both mechanisms.

The research in this volume contributes to the Aquafin CRC contract outcome: Support for new and emerging sectors of finfish aquaculture; Increased industry profitability through mastery of the quality requirements of the producer to market chain.

Effects of dietary astaxanthin and light manipulation

The first experiment was conducted to (1) evaluate the effects of increasing dietary concentrations of astaxanthin (supplied as Carophyll Pink™, Hoffmann-La Roche) and (2) examine the interactive effects of two forms of astaxanthin (unesterified Carophyll Pink™ or esterified Naturose™, Cyanotech Corporation) and shading on skin colour and astaxanthin content in the flesh and skin of snapper. Dietary levels between 36 and 72 mg astaxanthin kg^{-1} of either the unesterified (Carophyll Pink™) or esterified form (Naturose™) were adequate to increase the red colour in snapper skin during the experiment. Shading snapper from sunlight improved the lightness of their skin, resulting in more attractive fish, but was very difficult to achieve from a logistical perspective.

Effect of cage colour and light environment

To further investigate different methods to reduce the dark colour of snapper skin, the second series of experiments were conducted to examine the short-term effects of cage colour (black or white plastic-lined cages) and light environment (natural sunlight or reduced level of natural sunlight) on the skin colour of dark snapper and to examine the effect of those factors on post-mortem skin colour changes and marketability. One important question was whether excluding UV light would reduce the dark colour of snapper (in an attempt to mimic the attenuation of light wavelengths achieved at water depths of >several metres). The background colour of the cages had by far the most impact on skin colour and dark-coloured fish rapidly changed the lightness (L^*) of their skin after being exposed to white backgrounds. Exposing snapper to white backgrounds had the potential to improve the wholesale market price by between 10 and 50%. Reducing the intensity and altering the spectral profile of natural sunlight had no apparent effect on the L^* skin colour value.

If snapper are to be transferred to white or pale-coloured tanks before sale to increase skin lightness, a rapid turnover of fish is essential. An experiment was carried out to investigate the speed of the change in skin colour and plasma cortisol (an indication of stress) in response to a change in background colour. Rapid changes occurred in skin lightness after altering background colour with maximum change occurring within 1 day. Fish in this experiment were also fed diets containing astaxanthin and skin redness (*a** values) continued to steadily decrease over the 7 days when dark-coloured snapper were transferred to white backgrounds. Plasma cortisol concentrations were highest at stocking, when fish were held at greater densities, and were not affected by cage colour. The results of this study suggest that transferring dark-coloured snapper to a white background for 1 day is sufficient to affect the greatest benefit in terms of producing light-coloured fish while minimising the reduction in favourable red skin colouration.

Effects of carotenoids and background colour

A further series of experiments were conducted to determine the best type, concentration and duration of feeding of carotenoid pigment, and impacts of different cage colours (black, white, red or blue) on skin colour. Initially, we evaluated the effects of diets containing 60 mg kg^{-1} of either astaxanthin (Lucantin® Pink, BASF), canthaxanthin (Lucantin® Red), apocarotenoic acid ethyl ester (Lucantin® Yellow), selected combinations of the above, or no carotenoids, and holding snapper in either white or black plastic-lined cages. Diets containing astaxanthin conferred greatest skin pigmentation (*a**). Diets containing canthaxanthin led to a low level of deposition in the skin while apocarotenoic acid ethyl ester did not alter total skin carotenoid content or skin colour values in snapper. Snapper fed the astaxanthin diets were more yellow (higher *b** values) when held in red or white cages compared with fish held in black or blue cages despite similar feed intake and growth. The skin lightness (*L** values) of snapper was correlated with cage *L** values, with the lightest fish obtained from white cages. The results of these experiments indicate that snapper should be fed 30 mg astaxanthin kg^{-1} in white cages for 50 days to increase the red colouration prized in Australian markets. Subsequently, we demonstrated that 39 mg unesterified astaxanthin kg^{-1} (eg. as Lucantin® Pink) for 42 days was the optimal dietary level and duration of feeding to optimise skin pigmentation.

Effects of cage netting colour and density

Repeated results confirmed the benefits of transferring snapper to white backgrounds before harvest. However, this procedure would be expensive and it may be possible to use white netting cages in sea cage operations. In addition, the density of fish in cages may affect their perception and interaction with the colour of the cage surface. The experiments reported here were condcuted to (1) determine the effects of cage (net) colour on skin pigmentation, and (2) evaluate the effects of density on the skin colour and stress responses (cortisol – primary, and glucose – secondary) of snapper held in black and white plastic-lined cages. Skin lightness was improved without elevating plasma cortisol or glucose concentrations when snapper were held within solid-walled white surroundings for short periods, and is recommended over long-term growing in white netting sea cages prone to biofouling which masked the white colour required for background adaptation. While holding snapper in white cages at high densities greatly improves skin lightness in comparison to that of fish held in black cages, it is recommended that snapper be held at low densities to further enhance skin lightness.

Effects of cage colour and post-harvest K^+ concentration after storage
In addition to pre-harvest environmental manipulation and dietary carotenoids, skin colour can change post harvest. An experiment was conducted to examine the interactive effects of pre-harvest cage colour conditioning and short-term post-harvest immersion in K^+ (using potassium chloride, KCl) enriched seawater ice slurries on post-harvest skin colour. At harvest, there was no difference in skin lightness between fish held in white plastic-lined cages for 1 day or 7 days, but after storage on ice for 24 or 48 h, fish held previously in white cages for 7 days were significantly lighter (higher L^*). K^+ at 300-450 mmol L^{-1} made fish much lighter although this procedure is not recommended for short-term marketing because it also causes loss of the desirable red colour. If appropriate permits for use as an additive are obtained, K^+ can be supplied in seawater ice slurries for cultured snapper when stored for 24-48 h on ice for improved skin colour.

Final recommendations are to feed snapper diets fortified with 39 mg unesterified astaxanthin kg^{-1} for 6 weeks to enhance red pigmentation and then to hold fish before sale in white backgrounds for 1 day to maximise skin lightness.

KEYWORDS:
Astaxanthin; Dietary supplementation; *Pagrus auratus;* Skin colour; Snapper

1. BACKGROUND

This project formed part of the Research Program of the CRC for Sustainable Aquaculture of Finfish ("Aquafin CRC"), and employed funds invested out of the CRC's Commonwealth grant and by FRDC and other participants of the CRC.

When this project was conceived aquaculture of snapper, *Pagrus auratus*, in Australia is developing and commercial farms now operate in NSW and SA (Fielder, unpublished data). The potential for snapper aquaculture is demonstrated by the huge (approximately 50 000 t/yr) industry for this species in Japan. Although technology used in Japan formed the basis for early investigation of snapper culture in Australia, development of this infant industry, especially in NSW, has only been possible following research conducted by NSW Fisheries, FRDC ("Potential of snapper *Chrysophrys auratus* for aquaculture" (1989-1992)) and the CRC for Aquaculture (Project No. C4.2) to develop technology for broodstock management, intensive larval rearing, and evaluation of sea cages and inland saline ponds for growout, and diet development.

Some key differences between the industry in Australia and Japan have influenced technology transfer and the need for research described in this application. Most importantly, the market price for fish in Japan is approximately three times that received in Australia. This lessens the need to reduce production costs which is very important if snapper aquaculture is to reach its potential in Australia. Secondly, the hatchery sector in Japan is based on many generations of domestication. We found that just one generation of domestication with Australian snapper conferred major advantages in inducing spawning by manipulating photothermic regimes and expect that as subsequent generations are reared, advantages will compound. In general, hatchery production in Japan is based on greenwater systems. This is economically viable in Japan and even though extensive pond larval culture may be more cost-effective, many hatcheries in Japan have limited access to sufficient land for extensive ponds (land in general is very expensive in many areas in Japan where red sea bream hatcheries are located).

The red sea bream industry in Japan was built on feeding trash fish. It wasn't until the bait-fish industry in Japanese waters collapsed in the late 1980's and early 1990's that farmers were forced to replace trash fish. As late as 1995, commercial practices were still to blend frozen whole fish with a formulated premix. Diet development research in Japan since then has been conducted primarily by private feed companies and is generally not available in the technical or scientific literature.

In Australia, it is now possible to maintain captive broodstock snapper and to spawn high quality eggs on-demand, year-round. Also, intensive rearing of snapper larvae has been improved by identifying the optimum physical parameters such as photoperiod, salinity and temperature (Fielder and Allan, unpublished data). As a consequence, the time to rear snapper larvae to fully-weaned, metamorphosed fish has been reduced by approximately 2 weeks compared with the time taken using previous best-practice techniques. However, intensive larval fish rearing requires high capital and labour inputs as well as operation of facilities to culture live feeds such as rotifers and *Artemia*. These ancillary culture systems can be unreliable and expensive to operate. There is also a world shortage of *Artemia* cysts and purchase price has tripled in the last 12 months. Current industry estimates the cost of production of snapper at $1.00 /fingerling. In comparison, industry costs for producing other marine fingerlings are 34c for barramundi (Lobegeiger, 2001) and about 46c for bass. To improve profitability, there is an obvious need to develop techniques to reduce the cost and improve the vigour of fingerlings. Alternative hatchery methods or live foods and their replacement with artificial diets therefore needed to be identified.

Scientists at the Port Stephens Fisheries Centre (PSFC) linked with counterparts at SARDI who regularly breed large numbers of snapper for industry. Advances in larval rearing techniques have been validated at SARDI facilities. This has been an important conduit for technology transfer for South Australian hatcheries.

Extensive larval rearing, where ponds are fertilised to promote zooplankton populations (McCarty, Geiger, Sturmer, Gregg and Rutledge, 1986; Fielder, Bardsley and Allan, 1999), has been used to rear large numbers of several marine fish species such as red drum *Sciaenops ocellatus* (McCarty, *et al.* 1986), barramundi *Lates calcarifer* (Rutledge & Rimmer, 1991), Australian bass *Macquaria novemaculeata* (Battaglene, Talbot & Allan 1992) and mulloway *Argyrosomus japonicus* (Fielder, *et al.*, 1999). Growth and quality of fish can be high from fertilised ponds and cost of fingerling production can be low Because of the need for relatively unsophisticated facilities and low operating costs. However, survival of larvae can be variable Because of sub-optimal environmental conditions. In the successful FRDC Project No. 95/148, survival of mulloway larvae in fertilised ponds increased as the age of larvae that were stocked increased (Fielder, *et al.*, 1999). These results indicated that a combination of initial intensive rearing from hatch to consumption of *Artemia* (~14 d), when larvae are vulnerable to fluctuations in environmental conditions, followed by on-growing in fertilised ponds can optimise fingerling production. Preliminary trials during the CRC for Aquaculture project C4.2 demonstrated that snapper juveniles could be reared in commercial larval fish ponds; however, survival was low and the power of experiments was very low Because of low or no replication. These techniques may be suitable for large-scale production of cheap, high quality, healthy juvenile snapper but this must be verified in rigorous, replicated experiments.

Development of methods to reduce the reliance on *Artemia* as a live feed in intensive culture of juvenile snapper may also decrease the cost of production. Preliminary larval snapper rearing trials have demonstrated that *Artemia* can be replaced completely by extending the period of rotifer feeding and early addition of high quality imported weaning diets (Fielder, unpublished data). Indeed replacement of *Artemia* in aquaculture in Australia is viewed as a priority (McKinnon, Rimmer & Kolkovski, 2000) and FRDC have funded a project (2001-220) "Aquaculture Nutrition Subprogram: development of marine fish larval diets to replace *Artemia*" (PI Dr Sagiv Kolkovski), to investigate production of artificial microdiets for marine fish larvae. This technique has been evaluated in terms of fish quality, survival, reliability and cost of production. Also, replacement of *Artemia* with cultured copepods for marine fish larval rearing has recently attracted significant attention by larval fish culturists and was highlighted as a priority research area at the recent FRDC sponsored Live Feeds Workshop. As an alternative to intensive production of copepods (Rippingale, 1994), it may be possible to produce sustainable cultures of copepods in outdoor ponds, which could then be harvested and fed to snapper larvae in intensive tanks. Hundreds of thousands of juvenile Australian bass have been produced at the Port Stephens Fisheries Centre (PSFC) using this method, resulting in complete replacement of both rotifers and *Artemia*.

The research proposed in this application to decrease the cost of fingerling snapper has relevance to many other species of marine finfish. Feeding marine fish larvae is expensive and reducing mortality remains an international priority. Technologies developed and refined here will assist in efforts to reduce fingerling production costs for other species. Facilities at PSFC are unique as they include both large-scale production facilities and replicated smaller-scale facilities for larval rearing (intensive, semi-intensive and extensive). These types of facilities will be especially critical in the pursuit of successful tuna propagation and fingerling production.

New health management strategies are required to minimise the impact of disease in hatcheries. Disease outbreaks reduce vigour of fish and periodically cause excessive mortality resulting in

increased cost of fingerling production. Infestations of the ectoparasite *Amyloodinium* sp. in particular have caused significant mortality in Australian snapper hatcheries (Fielder & Allan, unpublished data) and is a major problem in overseas marine fish hatcheries (Paperna, 1983). Strategies to minimise losses include management to exclude or reduce the prevalence of disease, enhancement of fish resistance, and application of new methods of control.

A post-graduate Certificate student at the University of Queensland, Mr James Stopford, recently developed an excellent method to produce large number of *Amyloodinium* parasites in the laboratory. He operated his cultures for at least 10 generations and used the product to conduct preliminary investigations on the parasite. His work provided an excellent foundation for this project.

The cost of purchasing and delivering feeds is the single highest operating cost for most types of fish culture. To farm fish profitably, there is an obvious economic imperative to develop high-performance diets and feeding systems which are cost-effective. The diets also need to produce fish with desirable marketing traits. These traits include skin and flesh colour and flesh composition. To fetch premium prices, snapper need to have pink skin and white flesh. Large deposits of intestinal fat are perceived to be a marketing disadvantage. Fish are marketed as a “healthy” product, largely because fish fat has relatively high contents of the omega-3 highly unsaturated fatty acids. However, while replacing fish meal and fish oil in fish diets may reduce diet cost, and may also reduce P content in the effluent from farms, it will also reduce these health benefits.

There is also an important environmental imperative to minimise the amounts of nutrients and organic matter that are discharged from fish farms. To achieve these goals, diets need to satisfy but not oversupply essential nutrients and be made from high quality, highly digestible, readily obtainable ingredients. Diets also need to stimulate maximum consumption and deliver optimal feed conversion efficiency.

Excretion of nitrogen (N), phosphorus (P) and organic matter (mainly carbon, C) are the major pollutants from fish farms and their release is regulated by environmental protection agencies in many states in Australia. Feed is the sole source of these potential pollutants. To minimise N loss, N (protein) digestibility and retention must be increased. The recommended approach is to optimise protein quality and minimise diet protein content. This might be achieved by optimising the protein:energy ratio and using as much non-protein energy as possible. Strategies to minimise P loss include: selecting ingredients with high P bioavailability and selecting P supplements with high P absorbtion and low water solubility. To reduce organic matter pollution, diets must be highly digestible, promote maximum feed consumption and feed conversion efficiency. Minimising feed wastage through ensuring optimal pellet stability and determining the best feeding frequencies and feeding rates are critical factors in reducing pollution from fish farms.

FRDC currently fund snapper diet development research through project 99/323 "Aquaculture Diet Development (ADD) Subprogram: rapid development of diets for Australian snapper." Research under this project has determined:

1. Effects of shading and dietary astaxanthin source on skin colour. Results have demonstrated that although shading will reduce "skin blackness", shaded, farmed fish are still darker than wild fish and although shading is a viable option for some smaller, inshore fish farms, it is very difficult for large, offshore farms. To harvest genuinely "light" fish, farmers will need to find better ways to prevent melanisation. Addition of astaxanthin in either the free or esterified form increased the intensity of the "red" colour in the skin but this colour was not the highly desired "pink" of wild fish. More needs to be done to evaluate combinations of commercially available pigments to make fish pink and to reduce skin melanisation.

2. Identification of the best available commercial diet for snapper. Previous research under the ADD Subprogram for barramundi demonstrated the cost-effectiveness of nutrient-dense diets. It was expected that these would also benefit snapper but this has not been apparent in results from this commercial diet evaluation. Clearly, snapper have different nutrient requirements to barramundi, indicating that determination of their protein:energy requirements and ability to utilize carbohydrates for energy warrant independent investigation.

3. Protein:energy requirements will be determined at one temperature. This research will commence as soon as digestibility of experimental ingredients is completed.

4. Digestibility and utilization of key ingredients with potential to replace fishmeal and be incorporated into lower cost, high performance diets for snapper. This research is underway and will allow full investigation of digestibility of up to 16 ingredients and utilization of up to 8 ingredients.

5. Preliminary experiment to determine whether ionic deficiencies in inland saline water can be overcome through nutrition supplements. This research is planned to commence within six months.

The nutrition component of this research was fully integrated with FRDC 99/323. Already, the current FRDC project 99/323 addressed important questions and allowed formulation of better diets. However, diet development is an ongoing process. Increased funding for poultry and pig diet development is being allocated each year in recognition of the ongoing importance of feed and feeding to animal husbandry. This additional research on snapper diet development has proven to be a good investment for industry. The research on skin colour has identified the best mix of pigments and reduction of melanisation, beyond reduction possible using shading. Farmers have claimed they could receive up to an extra $3/kg for light and pink snapper. Research on alternative ingredients and on protein:energy requirements have been completed, in light of the apparent dissimilarity of snapper nutritional requirements to those of barramundi.

Research has also focussed on eco-friendly feeds that minimise concentration of N, P and C from uneaten or poorly utilized diets. One of the only ways to reduce N is to provide as much non-protein dietary energy as fish can utilize. The two non-protein dietary energy sources are lipid and carbohydrate. Utilization of these ingredients has been investigated and effects on body composition and taste determined.

Finally, recommendations have been made to optimize feeding strategies to minimize over-feeding.

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2. NEED

This project extended previous work, which demonstrated the feasibility of snapper farming in both marine and inland saline waters. It sought to reduce production costs by improving fingerling survival and growth and reducing input (feed) costs.

A reliable supply of cheap, high quality, healthy fingerlings is essential for development of viable snapper farming. When this project was conceived, industry estimated the cost of production of snapper at $1.00 per fingerling. This compares with less than 35¢ for barramundi fingerlings and about 46¢ for bass. To improve profitability, there is a need to reduce the cost and improve the vigour of fingerlings and to develop cost-effective high-performance diets and feeding systems for both hatchery and grow-out. This need has been recognised through the FRDC sponsored Hatchery Feeds RandD Plan (McKinnon *et al.*, 2000: http://www.aims.gov.au/hatchery-feeds), This project improved hatchery methods and replaced live feeds, such as brine shrimp (*Artemia*) whose supply and quality are unreliable, with alternative live feeds or artificial feeds. The project also developed better strategies for combining intensive and extensive rearing methods so as to optimise fingerling survival and quality. Research will have application for other species, including tuna.

Grow-out diets need to produce fish with desirable marketing traits, including colour. Fish are marketed as a "healthy" product, largely because fish fat has relatively high contents of the omega-3 highly unsaturated fatty acids. However, while replacing fishmeal and fish oil in fish diets may reduce diet costs, it will also reduce these health benefits. Minimising feed wastage through ensuring optimal pellet stability and determining the best feeding frequencies and feeding rates are critical factors in reducing pollution from fish farms. To achieve these goals, diets have been developed that satisfy but not oversupply essential nutrients and that are made from high quality, highly digestible, readily obtainable ingredients. Diets have been designed to stimulate maximum consumption and deliver optimal feed conversion efficiency. This research has built on successful results with snapper diet development under the previous FRDC ADD Subprogram snapper diet development project. The nutrition component of this project was fully integrated with the FRDC snapper diet development project.

Finally, the project sought to reduce disease-induced mortality by developing treatment methods for common parasites and establishing a foundation for immunological approaches to fish skin diseases.

3. OBJECTIVES

1. Improve production of snapper fingerlings by developing extensive, fertilised-pond rearing techniques for the advanced production of snapper juveniles.

2. Improve production of snapper fingerlings by developing larval feeding strategies to reduce the use of live feeds, in particular *Artemia*, by weaning larvae at an early age onto commercial and/or experimental artificial diets.

3. Improve production of snapper fingerlings by developing methods to reduce and/or treat the incidence of parasite infestation.

4. **Improve the skin colour of farmed snapper by reducing melanisation and improving skin pigmentation.**

5. Determine digestibility for, and ability of fish to utilize, new ingredients with potential for use in low-polluting snapper diets.

6. Evaluate ability of snapper to utilize carbohydrate and lipid sources for energy.

7. Determine optimum protein:energy ratio for fish grown at one favourable temperature.

8. Provide recommendations for feeding strategies to minimise overfeeding and maximise fish production.

This final report is published in three volumes. Objectives 5, 6 and 7 pertain to Volume 1: Diet Development; **Objective 4 pertains to this Volume 2**: **Skin Colour**; Objectives 1, 2, 3 and 8 pertain to Volume 3: Fingerling Production and Health.

4. RESULTS AND DISCUSSION

4.1 Effects of dietary astaxanthin source and light manipulation on the skin colour of Australian snapper *Pagrus auratus* (Bloch & Schneider, 1801)

Mark A. Booth[1], Rebecca Warner-Smith[1], Geoff L. Allan[1], and Brett Glencross[2]

[1] *NSW Department of Primary Industries, Port Stephens Fisheries Centre and Aquafin CRC, Private Bag 1, Nelson Bay NSW 2315*
[2] *Department of Fisheries, Government of WA, Fremantle Maritime Centre, 1 Fleet Street, Fremantle WA 6160*

ABSTRACT

Two experiments were conducted with Australian snapper *Pagrus auratus* (Bloch & Schneider, 1801). The first was aimed at determining the dietary level of astaxanthin that improved skin redness (CIE *a** values) of farm-reared snapper. Farmed snapper (ca. 600 g) fed a commercial diet without carotenoids were moved to indoor tanks and fed the same diet supplemented with 0, 36 or 72 mg astaxanthin kg^{-1} (unesterified form as Carophyll Pink™) for nine weeks. Skin redness (CIE *a** values) continued to decrease over time in fish fed the diet without astaxanthin. Snapper fed the diet containing 72 mg astaxanthin kg^{-1} were significantly more red than fish fed the diet with 36 mg astaxanthin kg^{-1} three weeks after feeding, but skin redness was similar in both groups of fish after six and nine weeks. The second experiment was designed to investigate the interactive effects of dietary astaxanthin source (unesterified form as Carophyll Pink™ or esterified form as NatuRose™; 60 mg astaxanthin kg^{-1}) and degree of shading (0, 50 and 95% shading from incident radiation) on skin colour (CIE *L*a*b**) and skin and fillet astaxanthin content of farmed snapper (ca. 800 g) held in 1 m^3 floating cages. After 116 days, there were no significant interactions between dietary treatment and degree of shading for *L*, a** or *b** skin colour values or the concentration of astaxanthin in the skin. Negligible amounts of astaxanthin were recovered from fillet samples. The addition of shade covers significantly increased skin lightness (*L**), possibly by reducing the effect of melanism in the skin, but there was no difference between the lightness of fish held under either 50 or 95% shade cover ($P>0.05$).

1. INTRODUCTION

Australian snapper (*Pagrus auratus*) is a premium table fish that fetches high market prices in eastern Australia. The species has previously been reported as *Chrysophrys auratus* and *C. unicolour* in Australasia; and *Pagrus major* and *C. major* in Indo-China-Japan, where it is commonly known as red sea bream (Paulin, 1990). The capture fishery for this species in Australia has declined by 50% over the past decade (Quartararo, 1996), and between 1000 and 2000 t are now imported annually. This decline, along with the success of the red sea bream industry in Japan, has prompted the development of a snapper aquaculture industry in Australia. A small number of commercial snapper farms now operate in South Australia and New South Wales. However, farmed snapper are generally darker and less red in appearance than those obtained from the wild harvest. This difference has economic consequences for producers of farmed snapper, with their product often being bought and sold at discounted prices. Parallels can be drawn between consumer demand and preference for the intense red pigmentation of salmon, with higher prices paid for highly pigmented flesh (Simpson & Kamata, 1979; Torrissen, Christiansen, Struksnaes & Estermann, 1995).

Wild-caught Australian snapper are characteristically identified by the bright pink/red pigmentation of their skin (Last, Yearsley & Ruello, 1999). This colour is presumably Because of the deposition of carotenoids (specifically astaxanthin; 3,3'-dihydroxy-β,β-carotene-4,4'-dione) from their natural food sources. As snapper are unable to form or convert intermediary precursor pigments to astaxanthin (Katayama, Shintani, Shimaya, Imai & Chichester, 1972; Tanaka, Katayama, Simpson & Chichester, 1976; Nakazoe, Ishii, Kamimoto & Takeuchi,. 1984), diets must be supplemented with astaxanthin for snapper to attain the characteristic pink/red colour of wild specimens (Nakazoe, *et al.* 1984). The dark skin colour of farm-reared fish may be a response to the proximity of sea cages to the surface of the water and the subsequent effect of sunlight exposure on melanin concentration in the skin. In farms where floating cages are used, the use of shade cloth covers or similar has been shown to reduce melanism (Matsui, Tanabe, Furuichi, Yoshimatsu & Kitajima, 1992; Quartararo, 1996).

The use of astaxanthin in the diets of Australian snapper and the provision of shade covers may improve the skin colour of farmed snapper. Therefore, the major aims of this study were to (1) evaluate the effects of increasing dietary concentrations of unesterified astaxanthin (Carophyll Pink™) on skin colour of snapper and (2) study the interactive effects of unesterified (Carophyll Pink™) or esterified (Naturose™) forms of astaxanthin and shading on skin colour and astaxanthin content in the flesh and skin of snapper.

2. MATERIALS AND METHODS

2.1 Tristimuli colourimetric analysis

The skin colour of snapper was measured using either a CR-10 or CR-300 hand held Chromameter (Minolta, UK: Selby Biolab, Gladesville, NSW) and colour was quantified according to the $L^*a^*b^*$ colour space (CIE, 1976). The L^*, a^* and b^* characteristics on the left flank of each fish was measured at three positions; above the pectoral fin immediately below the lateral line, above the anus and on the caudal peduncle. The three colour readings were then averaged to provide an individual colour assessment for each fish [e.g. $\bar{a}^* = (a^*+a^*+a^*)/3$].

2.2 Effect of dietary astaxanthin content on skin colour: Experiment 1

This experiment was carried out to determine the effect of different dietary levels of Carophyll™ Pink (min. 8% astaxanthin; F. Hoffmann-La Roche Ltd, Basel, Switzerland) on skin colour of snapper. Sixty, 18 month old (mean 609 ± 127.1 g) fish were obtained from a research sea cage near Geraldton, Western Australia (Central West TAFE, Geraldton, WA, Australia, S 32°40'22'', E 152°13'04). Fish were previously fed a commercial snapper diet with no added astaxanthin (40% CP, GlennForrest Stockfeeders, Glenforrest, WA, Australia). The fish were placed in seawater with anaesthetic (20 mg L^{-1} clove oil), before being tagged with a small (12 mm length) dart tag to allow identification of individuals through time and then each fish was allocated to an experiment tank. Ten fish were kept in each of six, indoor, 1000 L tanks in a re-circulating seawater system (Maritime Centre, Fremantle, WA, Australia). Ambient light levels were considered low, but were not measured. Partial exchange of seawater took place once weekly.

Three experimental diets were made by grinding the "Glenforrest" diet and adding Carophyll Pink™ to give dietary levels of 0, 36 and 72 mg astaxanthin kg^{-1}. The diets were re-pelleted using a steam pellet press with a 4 mm die. Two experiment tanks were randomly allocated to each of the test diets and fish were fed once daily at about 1.5% of their body weight per day for nine weeks.

Skin colour readings were taken with a Minolta CR-10 at stocking and once every three weeks on fish that had been anaesthetised.

2.3 Effect of astaxanthin source and shading on skin colour and skin or fillet astaxanthin content of snapper: Experiment 2

This experiment was designed to investigate the interactive effects of two sources of astaxanthin and three types of shading on the skin colour and astaxanthin concentration in the skin and fillet tissue of snapper. Each of the nine treatments was replicated 4 times. Astaxanthin was supplied either as Carophyll Pink™ or NatuRose™ (min. 1.5% astaxanthin; Cyanotech Corporation, Hawaii, USA). Carophyll Pink™ is a chemically synthesised "nature identical' form of astaxanthin in which the total carotenoid composition is dominated by mostly all-trans astaxanthin (73%) and cis astaxanthin (18%). The remainder is made up of astacene, canthaxanthin and lutein (Gomes, *et al*. 2002). NatuRose™ is produced from Haematococcus algae meal and its carotenoid composition closely resembles that of krill, shrimp and crayfish (Lorenz, 1998). It provides astaxanthin predominantly in an esterified form with the carotenoid fraction of the meal consisting of about 70% monoesters of astaxanthin, 10% diesters of astaxanthin, 5% unesterified astaxanthin and the rest as β-carotene, canthaxanthin, lutein and other carotenoids (Lorenz, 1998).

The experiment was conducted at a commercial fish farm (Kurnell, NSW, Australia) using two-year-old Australian snapper (c.a. 800 g) obtained from the farm. Before the trial, fish were fed a commercial diet (43% CP; Pivot Aquaculture Pty. Ltd, Cambridge, Tasmania, Australia) and held in shade-cloth covered cages (approximately 50% shade). Three experimental diets were made by grinding the "Pivot" diet. Two diets were supplemented with 60 mg astaxanthin kg^{-1} of Carophyll™ Pink or NatuRose™ plus an additional 5% to account for processing losses (following manufacturers' recommendations). The third diet, hereafter known as the basal diet, had no pigment added, but was otherwise treated similarly to the pigmented diets. Each mash was reformed into 9 mm diameter pellets using a steam pellet press (temperature ≤ 65.2°C; 500-B Junior Ace Pellet Mill, Sprout-Waldron, Pennsylvania, USA). Snapper were hand fed test diets at approximately 2% of their body weight once a day by the farm staff.

The farm provided 3 x 25 m^2 pontoons that had space for 12 x 1 m^3 experiment cages to be fixed to the inner perimeter of each pontoon. Each of the 36 cages was randomly positioned within each pontoon, but each pontoon was assigned at least one cage per treatment. Experiment cages were constructed of 9 mm knotless mesh stretched over PVC frames. Shaded cages (50 and 95% shade) were covered on four sides and the top with a removable cover attached by velcro fasteners. The experimental cages and shade covers were exchanged every two weeks to prevent the reduction of water flow or light penetration Because of bio-fouling. Greater than 95% shade was provided by black plastic woven 'weedmat', and 50% shade was provided by black plastic woven 'sarlon' shade cloth (Agrifab, Pty. Ltd., Mortlake, NSW, Australia).

Visible light intensity was measured as photosynthetically active radiation (PAR) in each cage on three separate, cloudless days (between 1100 and 1300 h). A LI-188B Quantum/Radiometer/Photometer and a LI-192SB Underwater Quantum Sensor (LI-COR, Lincoln, Nebraska, USA) were used to measure PAR, with the sensor lowered to within 15 cm of the base of each cage. An average value for each level of the "shade" factor was calculated from 12 cages. Average values (mean ± sem, n= 3 days) were 275.55 ± 32.72 μE s^{-1} m^{-2}, 100.71 ± 5.02 μE s^{-1} m^{-2}, and 7.64 ± 3.22 μE s^{-1} m^{-2} for 0%, 50% and greater than 95% shade respectively.

The skin colour of all fish stocked into cages was recorded at the start (Minolta CR-10) and end (Minolta CR-300) of the experiment. To account for differences between the instruments, 4 standard colour tiles were compared. The relationship between the absolute values for each coordinate (*L*, a*, b**) was then determined using linear regression analysis and used to standardise the final skin colour readings. At the end of the experiment, the skin and flesh of three randomly selected fish from each

cage was removed for carotenoid analysis. Extraction of carotenoids from fish skin or flesh followed similar techniques to those described by Dall (1995), with the exception that sample preparation proceeded only far enough to allow determination of total carotenoids by visual spectroscopy (Varian DMS 100S, Chatswood, NSW, Australia). The carotenoid concentration of skin and tissue samples was calculated using $E_{1\%,\ 1cm} = 2100$ for astaxanthin in hexane and measuring optical density (OD) at λ_{max} 470 nm.

2.4 Statistical analysis

One-way ANOVA was used to investigate the effect of astaxanthin level (supplied as Carophyll Pink™) on the skin colour of snapper in experiment 1. Experiment 2 was originally designed for investigation using a fully orthogonal two-way ANOVA. However, four cages, two from the greater than 95% shade treatment fed Carophyll Pink™, one from the 50% shade treatment fed Carophyll Pink™ and one from the 50% shade treatment fed NatuRose™, were lost during a severe storm. Consequently, an unbalanced two-way ANOVA was performed on selected response variables. The level of significance for all tests was set at $\alpha = 0.05$ and the Student Newman-Keuls multiple comparison test was used to discriminate between means. All statistical analyses were performed using Statgraphics Plus V4.1 (Manugistics, Inc. Rockville, USA).

3. RESULTS

3.1 Effect of dietary astaxanthin content on skin colour: Experiment 1

There were no significant differences between the *L**, *a** or *b** skin colour values of snapper assigned to each of the three dietary treatments at the beginning of the experiment. Dietary treatment did not affect *L** or *b** skin colour values after 3, 6 or 9 weeks of feeding (Table 1). However, *a** skin colour values differed between dietary treatments after 3, 6 and 9 weeks of feeding, respectively (Table 1). The *a** value of fish fed on diets with 36 and 72 mg kg^{-1} unesterified astaxanthin (Carophyll Pink™) remained at almost the same level as that of fish measured at the beginning of the experiment, while the *a** value of fish fed the non-supplemented diet decreased.

3.2 Effect of astaxanthin source and shading on skin colour and skin or fillet astaxanthin content: Experiment 2

Initial means for skin colour (*L**, *a** or *b** values) did not differ between treatments. At the completion of the experiment, there was no significant interaction between diet and shade for either *L**, *a** or *b** skin colour values. Shade but not diet significantly affected *L** and *b** skin colour values and both diet and shade significantly affected *a** skin colour values (Table 2). Snapper held in shaded cages were significantly lighter (more positive *L** value) than snapper held in unshaded cages. Although there was no significant difference between the lightness of snapper held in shaded cages, snapper held under the 95% shade treatments tended to be slightly lighter than those held under 50% shade (Table 2). Snapper fed diets containing astaxanthin were significantly redder (higher positive *a** value) than snapper fed diets without supplemental astaxanthin, however there was no significant difference between the redness of snapper fed diets supplemented with either Carophyll Pink™ or NatuRose™. The *a** value of snapper skin decreased significantly as the level of shading was increased.

Both diet and shade, but not their interaction, affected the skin astaxanthin content of snapper (Table 3). However, the astaxanthin concentration in selected tissue samples was negligible and was not found at levels > 0.02 mg 100 g^{-1}. Fish fed diets supplemented with either Carophyll Pink™ or NatuRose™ had significantly higher skin astaxanthin contents than those fed the basal diet, but the skin astaxanthin content of fish fed the supplemented diets was not significantly different from each other. Snapper held

without shade had significantly higher skin astaxanthin contents than those held under 50% or 95% shade cover. Post-hoc regression analysis illustrated a reasonably strong logarithmic relationship between the treatment means (n=9) of astaxanthin concentration in the skin and instrumentally measured a^* skin colour values (a^* = 12.73 + 3.49 x ln (astaxanthin concentration of skin; R^2 = 0.80). Removal of one outlier Because of an unusually high studentised residual (>3.0) improved the goodness of fit (a^* = 13.11 + 3.80 x ln (astaxanthin concentration of skin; R^2 = 0.96).

4. DISCUSSION

The optimal level of astaxanthin required to illicit a skin colour response in snapper was not determined, but inclusion of 36 or 72 mg kg^{-1} of astaxanthin (Carophyll Pink™) in a commercial feed was effective at maintaining the initial a^* skin colour value of fish from the Geraldton farm. The fact that snapper fed the non-supplemented diet became less red with time suggests they may have been consuming organisms containing astaxanthin before being moved indoors and confirms that the control diet did not contribute to skin redness. Although this trial was only run for a short period, a^* skin colour values of snapper fed the supplemented diet were similar by week 6 and remained so after week 9. This suggests that some type of biological saturation may have been reached under the feeding regime we adopted. Use of a^* skin colour values alone would not be enough to support this hypothesis, but the strong relationship we found between a^* skin colour values and the measured astaxanthin content of the skin of snapper suggests the link may be plausible. Ito, Kamata, Tanaka and Sameshima (1986) found that the concentration of astaxanthin in skin samples of red sea bream *P. major* fed diets supplemented with 100 mg esterified astaxanthin kg^{-1} plateaued after one months feeding. Despite this level being much higher than that tested in either of our experiments, the astaxanthin content of the skin of red sea bream was almost identical to that of snapper in our study. Similarly, Gomes, Dias, Silva, Valente, Empis, Gouveia, Bowen and Young (2002) found that the astaxanthin concentration in the skin of gilthead seabream *Sparus auratus*, another closely related species, plateaued after 3 weeks feeding on diets containing 40 mg kg^{-1} astaxanthin as Carophyll Pink™. Although optimal astaxanthin inclusion content was not determined, given the high cost of carotenoids, it is clear from our results that both feed concentration, feed intake and length of feeding time are variables which require further research to optimise.

The addition of shade covers to 1 m^3 floating cages significantly increased the lightness (L^*) of snapper skin. This is likely Because of a reduction in melanism as a result of reduced exposure to the sun. There is some evidence to suggest that the particular transmission qualities of some materials can lead to improvements in the lightness of fish. Matsui, *et al.* (1992) reported that shading red sea bream from sunlight using either agricultural shade cloth or thick black vinyl sheeting maintained the L^* value of fish at similar levels to those of wild controls (approximate L^* values between 55-60). However, these authors also found that fish held under a vinyl sheet that excluded only wavelengths below 390 nm and transmitted more than 80% of natural sunlight became significantly darker than wild controls and were similar to fish held without any shade cover after 84 days (approximate L^* values between 38-40). This suggests that wavelengths above the UV range or the intensity of radiation are important factors affecting the lightness of farmed snapper held in cages.

True sunburn (erythema) in fish as a result of UV exposure is characterised by lesions on the dorsal surface and erosion of the epidermis as well as the formation of cataracts on the eyes (multiple authors cited in Zagarese & Williamson, 2000). None of these signs were observed in any of the fish used in our study. Besides shading, other methods have been employed to improve the lightness of snapper (increase L^*). Lin, Ushio, Ohshima, Yamanaka and Koizumi (1998), recommended the immersion of cultured snapper in low temperature soaking solutions of 300 mmol L^{-1} K+ immediately after death to induce the aggregation of melanosomes in skin melanophores, preventing the natural darkening of the skin that occurred after death.

The inclusion of unesterified (Carophyll Pink™) or esterified (NatuRose™) forms of astaxanthin significantly increased the carotenoid content in the skin of snapper and the measured values from our study were comparable to those presented for red sea bream fed astaxanthin in other studies (Nakazoe, *et al.* 1984; Ito, *et al.* 1986; Ha, Kang, Kim, Choi & Ryu, 1993). We found no difference in the skin astaxanthin content of snapper fed either of the supplements, but others have reported that red sea bream utilise esterified forms of astaxanthin more efficiently than unesterified forms (Nakazoe, *et al.* 1984; Ito, *et al.* 1986). Gomes *et al.* (2002) found no difference between the skin astaxanthin content of gilthead sea bream fed Carophyll Pink™ or Naturose™ and like this study, found theses sources of astaxanthin did not affect (i.e. increase) muscle carotenoid content. While it is clear that both sources of astaxanthin have increased the red pigmentation in the skin of 800 g snapper, the fact that feed intake, the astaxanthin content of the test diets and the digestibility of each astaxanthin source was not determined precludes us from discussing the efficacy of either source.

Our studies indicate that inclusion of astaxanthin in the diets of farmed Australian snapper can improve skin redness. The concentration required and length of time necessary to induce an improvement in skin colour was not determined, but dietary levels between 36 to 72 mg astaxanthin kg^{-1} of either the unesterified (Carophyll Pink™) or esterified form (Naturose™) were adequate to induce treatment effects during the course of both short and longer-term trials reported in this paper. Shading snapper from sunlight appears to have improved the lightness of their skin resulting in more attractive specimens, but the practicalities of screening large sea cages needs further investigation. Further manipulation and investigation of these parameters should help reduce the disparity in appearance of snapper grown in open sea cages with those caught from the wild.

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TABLE 1

*L*a*b** skin colour values of Australian snapper *Pagrus auratus* held indoors and fed diets containing 0, 36 or 72 mg kg^{-1} astaxanthin (Carophyll Pink™) supplements for nine weeks.

		Dietary level of astaxanthin mg kg^{-1}		
		0	36	72
Week	Coordinate	Skin colour value		
0	*L**	48.9 ± 2.9	45.8 ± 0.6	49.3 ± 1.5
	*a**	8.8 ± 0.4	7.9 ± 0.3	8.0 ± 0.0
	*b**	2.8 ± 0.5	2.7 ± 0.6	2.5 ± 0.2
3	*L**	49.9 ± 1.0	46.8 ± 1.3	46.4 ± 1.3
	*a**	5.2±0.2^{a}	6.5 ± 0.2^{b}	7.8 ± 0.1^{c}
	*b**	3.8±0.1	5.5 ± 0.2	5.8 ± 0.8
6	*L**	49.4 ± 3.4	48.1 ± 0.2	48.5 ± 0.6
	*a**	4.6 ± 0.1^{a}	8.0 ± 0.1^{b}	7.9 ± 0.1^{b}
	*b**	2.5 ± 0.5	2.5 ± 0.0	2.6 ± 0.2
9	*L**	51.1 ± 2.1	53.3 ± 4.6	51.7 ± 0.1
	*a**	4.0 ± 0.5^{a}	7.7 ± 0.4^{b}	8.5 ± 0.2^{b}
	*b**	8.8 ± 1.2	9.2 ± 0.5	9.6 ± 0.6

Values are mean ± standard error of two replicate tanks. Significant differences between the same coordinate within the same week are indicated by different letters in superscript (one-way ANOVA; SNK, $P<0.05$)

TABLE 2

*L*a*b** skin colour values of Australian snapper *Pagrus auratus* held in shaded cages and fed diets containing no astaxanthin or diets containing Carophyll Pink™ or NatuRose™.

Main effect	Level	Skin colour value[1]		
		*L**	*a**	*b**
Diet	Basal	62.3 ± 1.0	9.2 ± 0.2^{x}	13.9 ± 0.3
	Carophyll Pink™	59.4 ± 1.2	11.9 ± 0.2^{y}	14.6 ± 0.4
	NatuRose™	61.6 ± 1.0	12.2 ± 0.2^{y}	14.7 ± 0.4
Shade	0	58.3 ± 1.0^{a}	12.0 ± 0.2^{c}	14.4 ± 0.3^{ab}
	50	61.6 ± 1.1^{b}	11.2 ± 0.2^{b}	15.3 ± 0.4^{b}
	95	63.4 ± 1.1^{b}	10.0 ± 0.2^{a}	13.5 ± 0.4^{a}

[1]Values are level means ± standard error (two-way ANOVA). For each factor, significant differences (P<0.05) between level means are indicated by different superscript letters. The grand mean ± pooled standard error for the initial skin colour of snapper was *L**=62.9±1.3; *a**=5.8±0.7; *b**=15.2±0.5.

TABLE 3

Astaxanthin concentration in the skin of Australian snapper *Pagrus auratus* held in shaded cages and fed diets containing no astaxanthin or diets containing Carophyll Pink™ or NatuRose™.

Main effect	Level	Astaxanthin concentration in skin[1] (mg 100 g^{-1})
Diet	Basal	0.392 ± 0.050^{x}
	Carophyll Pink™	0.844 ± 0.060^{y}
	NatuRose™	0.784 ± 0.053^{y}
Shade	0	0.806 ± 0.050^{b}
	50	0.600 ± 0.055^{a}
	95	0.615 ± 0.058^{a}

[1]Values indicate level means ± standard error (two-way ANOVA). For each factor, significant differences (P<0.05) between level means is indicated by different superscript letters. Astaxanthin levels in fillet tissue were negligible.

4.2 Effect of cage colour and light environment on the skin colour of Australian snapper, *Pagrus auratus* (Bloch & Schneider, 1801)

Ben J. Doolan[1,2], Mark A. Booth[2], Paul L. Jones[1] and Geoff L. Allan[2]

[1] *School of Life and Environmental Sciences, Deakin University, Warrnambool, Victoria 3280, Australia*
[2] *NSW Department of Primary Industries, Port Stephens Fisheries Centre and Aquafin CRC, Private Bag 1, Nelson Bay NSW 2315*

ABSTRACT

A two-factor experiment was performed to evaluate the effects of cage colour (black or white 0.5 m^3 experiment cages) and light environment (natural sunlight or reduced level of natural sunlight) on the skin colour of darkened Australian snapper. Each treatment was replicated four times and each replicate cage was stocked with five snapper (mean weight = 351 g). Snapper exposed to natural sunlight were held in experimental cages located in outdoor tanks. An approximately 70% reduction in natural sunlight (measured as PAR), was established by holding snapper in experiment cages that were housed inside a 'shade-house' enclosure. The skin colour of anaesthetised fish was measured at stocking and after a 2-, 7- and 14-day exposure using a digital chroma-meter (Minolta CR-10) that quantified skin colour according to the *L*a*b** colour space. At the conclusion of the experiment, fish were killed in salt water ice slurry and post-mortem skin colour quantified after 0.75, 6 and 22 h respectively. In addition to these trials, an *ad hoc* market appraisal of chilled snapper (mean weight = 409 g) that had been held in either white or black cages was conducted at two local fish markets.

Irrespective of sampling time, skin lightness (*L**) was significantly affected by cage colour ($P < 0.05$), with fish in white cages having much higher *L** values ($L^* \approx 64$) than fish held in black cages ($L^* \approx 49$). However, the value of *L** was not significantly affected by the light environment or the interaction between cage colour and the light environment. In general, the *L** values of anaesthetized snapper were sustained post-mortem, but there were linear reductions in the *a** (red) and *b** (yellow) skin colour values of chilled snapper over time. According to the commercial buyers interviewed, chilled snapper that had been reared for a short period of time in white cages could demand a premium of 10–50% above the prices paid for similar sized snapper reared in black cages. Our results demonstrate that short-term use of white cages can reduce the dark skin colour of farmed snapper, potentially improving the profitability of snapper farming.

1. INTRODUCTION

The skin colour of Australian snapper *Pagrus auratus* (i.e. *P. auratus* = *Pagrus major*; Tabata & Taniguchi, 2000), grown in sea cages is darker than wild snapper harvested from commercial fisheries. The dark appearance of farmed snapper also tends to worsen after they are harvested and chilled. This difference has negatively affected the market price of farmed snapper because many consumers find their appearance displeasing. The disparity between the skin colour of harvested versus wild fish has also been documented in other sparids including the Japanese red sea bream *P. major* (Hatae, 1990; Matsui, Tanabe, Furuichi, Yoshimatsu & Kitajima, 1992; Fujii, 1993; Lin, Ushio, Ohshima, Yamanaka & Koizumi, 1998a, b) and red porgy *P. pagrus* (Szisch, van der Salm, Wendelaar Bonga & Pavlidis, 2002; Rotllant, Tort, Montero, Pavlidis, Martinez, Wendelaar Bonga & Balm; Balm, 2003; Van der Salm, Martinez, Flik & Wendelaar Bonga, 2004; Chatzifotis, Pavlidis, Jimeno, Vardanis, Sterioti & Divanach, 2005).

The cause of skin darkening in farmed fish is generally unknown, but factors such as stress (Rottlant, *et al.* 2003), background colour (Rotllant, *et al.* 2003; Van der Salm, *et al.* 2004) and exposure to incident solar radiation (Matsui, *et al.* 1992; Szisch, *et al.* 2002; Booth, Warner-Smith, Allan & Glencross, 2004 [Chapter 4.1]); Van der Salm, *et al.* 2004) have been implicated. These stressors are thought to act upon the neuro-endocrine system, resulting in the release of a suite of hormones that affect various chromatophores situated in the skin. Centrifugal translocation (i.e. dispersal) of pigmentary organelles known as melanosomes within dermal melanophores causes darkening of the skin, whereas centripetal migration (i.e. aggregation) causes the skin to appear lighter (see the review by Fujii and Oshima, 1986).

Recently, Booth, *et al.* (2004 [Chapter 4.1]) demonstrated that the skin colour of farmed snapper could be lightened by covering the top and walls of small experiment cages (1 m^3) with a black shade-cloth, which reduced the level of natural incident solar radiation. Other studies have reported on the effectiveness of employing white tanks to enhance the skin lightness of red porgy (Rotllant, *et al.* 2003; Van der Salm, *et al.* 2004). Wakazono, Okajima, shirai, Kitajima, Matsui, Tanabe and Matsumoto (1992) suggested that UV–B radiation might play a role in melanogenesis in red sea bream; however, evidence to the contrary was presented by Matsui, *et al.* (1992), who found that farmed red sea bream held under vinyl sheeting that excluded UV wavelengths <390 nm, but which transmitted more than 80% of visible sunlight, became significantly darker than wild controls and had lightness (*L** value) similar to fish held without any shade cover. The treatments used in the different studies obviously altered both the spectral and quantal profile of the incident light reaching the fish; however, the specific wavelengths and intensities involved are poorly elucidated.

NSW DPI Fisheries is presently engaged in research aimed at improving the skin colour of Australian snapper grown in sea cages. This research focuses on several aspects pertinent to the culture of these fish including the addition of carotenoids to feeds and the manipulation of their physical environment. The present paper concentrates primarily on environmental methods that reduce the dark skin colour of farmed snapper. As such, it examines the short-term effects of cage colour (black or white cages) and light environment (natural sunlight or reduced level of natural sunlight) on the skin colour of artificially darkened snapper. In addition, the effect of these factors on post-mortem skin colour changes and marketability are presented.

2. MATERIALS AND METHODS

2.1 Measurement of colour

The skin colour of snapper and the colour of experimental tanks and cages were quantified using a hand held chroma-meter (Minolta CR-10 Color Reader; Minolta, UK: Selby Biolab, Gladesville, NSW, Australia). Colour was quantified according to the Commission Internationale de l'Éclairage (CIE) *L*a*b** colour space. In this colour space, *L** represents lightness (black 0 to white 100), *a** represents green (-60) to red (+60), and *b** represents blue (-60) to yellow (+60). *L*a*b** tristimulus colour measurements have been used previously to quantify the skin colour of several *Pagrus* spp. (e.g. Guerin, 2001; Guerin & Hosokawa 2001; Booth *et al.* 2004, Chapter 4.1); Van der Salm *et al.* 2004; Kalinowski, Robaina, Fernández-Palacios, Schuchardt & Izquierdo, 2005).

The skin colour of each fish was quantified by taking 3 individual skin colour readings along the lateral line: behind the operculum, beneath the dorsal fin and on the caudal peduncle. The *L**, *a** and *b** readings from each point were then averaged to give a representative skin colour value for each fish (Booth *et al.* 2004 [Chapter 4.1]).

2.2 Pre–treatment of snapper

In order to darken all snapper before the experiment, they were held for 28 days in an outdoor tank (9 kL) fitted with a black PVC liner (L^* = 28.2, a^* = -0.7, b^* = 1.3). This outdoor tank was part of a larger saltwater recirculating aquaculture system (RAS) at the Port Stephens Fisheries Centre (PSFC). During this period the 9 kL tank was exposed to approximately 14 h of natural sunlight per day (photoperiod 14L:10D) and the snapper were fed 0.5% BW day^{-1} of a commercial barramundi *Lates calcarifer* feed devoid of additional carotenoids (45% crude protein, 20% fat).

2.3 Experimental tank systems

Two separate, saltwater RAS were used to house the experiment cages. The first system was located outdoors in direct sunlight (as described above) and consisted of two 9 kL circular tanks (3.4 m diameter x 1.0 m depth) fitted with black PVC liners. The second saltwater RAS was located inside a 'shade-house' adjacent to the aforementioned system. This system consisted of two 10 kL circular fibreglass tanks (3.4 m diameter x 1.1 m depth; L^* = 75.1, a^* = -12.6, b^* = -1.9). Each of the tanks in both systems was aerated using 2 x 100 mm air stone diffusers supplied with compressed air. Regular water exchange was facilitated by vacuum siphoning each tank every third day to remove accumulated solids and uneaten feed.

Suspended in each tank at each location were two black and two white experiment cages (1.0 m x 1.0 m x 0.5 m) made of a 9 mm knotless mesh fixed to a rigid, rectangular PVC frame. Cage depth was set to 0.5 m to ensure snapper remained close to the water surface. Cages were fully lined with either black (L^* = 22.3, a^* = 0.6, b^* = 1.1) or white (L^* = 90.8, a^* = 1.8, b^* = 0.8), 320 µm PVC sheeting (Newcastle Plastics, Islington, NSW, Australia) except for a narrow 100 mm strip at the bottom of each cage, which facilitated water exchange and waste removal. Each experimental cage was also fitted with a simple airlift pump to ensure that adequate levels of dissolved oxygen were maintained through additional water exchange (5–6 L min^{-1}). All cages were covered with a black, open weave (25 mm) bird netting to prevent the escape of fish. Water quality was monitored daily and remained within acceptable limits for marine finfish.

2.4 Reduction in natural level of incident sunlight

The shade-house covering the indoor RAS consisted of a double layer of translucent 150 µm polyethylene sheeting fixed securely beneath an outer layer of woven white broad-spectrum shade cloth (Floraguard Pty. Ltd., Box Hill, NSW, Australia). The water surface of the experiment tanks inside the shade-house was approximately 2.0–3.0 m below the plastic sheeting.

Samples of the materials covering the shade-house were removed and mounted on a rigid cardboard frame in order to determine their spectral transmission qualities. Spectral transmittance was measured on a Varian DMS 100S Spectrophotometer (Varian, Chatswood, NSW, Australia) using an automated scanning process covering wavelengths from 200 to 700 nm (Rothery, 1982). The resultant scan indicated that the double layer of plastic in combination with the woven shade cloth altered the intensity as well as the spectral composition of light transmitted to the experiment tanks (Figure 1).

The intensity of photosynthetically active radiation (PAR) reaching fish held in both light environments (i.e. locations) was quantified using an LI-188B Quantum/Radiometer/Photometer fitted with an LI-192SB Underwater Quantum Sensor (LI-COR, Lincoln, NE, USA). Photosynthetically active radiation was measured at the air/water interface and at 0.05 m and 0.50 m below the water surface on five randomly selected, cloudless days at approximately 12:00 hours (Australian Eastern Standard Time). The average PAR (reported as mean ± SEM) recorded at the respective depths in the

first experiment was 1713 ± 18, 1528 ± 16 and 1220 ± 50 µE s^{-1} m^{-2} (outdoor tank system) and 527.2 ± 14.9, 491 ± 20.9, and 339 ± 8.9 µE s^{-1} m^{-2} (shade-house tank system). This equates to an approximately 69, 68, and 72% reduction in PAR at the three depths, respectively.

2.5 Effect of cage colour and light environment on skin colour – part A

Darkened snapper were lightly anaesthetized in the black tank (10 mg L^{-1} ethyl-p-aminobenzoate), captured in small groups and transferred to an aerated plastic tub (200 L) that contained a heavier dose of anaesthetic (20–25 mg L^{-1} ethyl-p-aminobenzoate. Once fish were unconscious, the skin colour of individual snapper was recorded (L^* = 51.5 ± 4.2, a^* = 5.1 ± 1.1, b^* = 8.3 ± 1.6; mean ± SD, n = 79). Fish were then weighed (351 ± 72 g; mean wet weight ± SD), fin-clipped and systematically distributed to each of the 16 experimental cages until each cage contained five snapper. The skin colour of each fish was re-recorded 2, 7 and 14 days after stocking by anaesthetizing fish in situ. On each occasion, the cages were thoroughly cleaned before fish were returned.

2.6 Effect of cage colour and light environment on post-mortem skin colour – part B

In order to determine post-mortem treatment effects on the skin colour of snapper, two fish were rapidly removed from black or white experimental cages without anaesthesia on day 16. Fish were killed using a technique similar to that practiced by commercial fish growers; i.e. individual fish were removed from cages using a large dip net and rapidly transferred to a -1.0ºC saltwater ice slurry (31 g L^{-1}). Once immersed and after the fish became motionless, they were removed to attach a waterproof label to the caudal fin to ensure traceability. After labelling, each fish was re-immersed in the ice slurry for 0.75 h before post-mortem skin colour values were recorded. After immersion, fish were removed from the ice slurry, washed and packed into polystyrene boxes. Fish were covered in ice and stored overnight in the dark in an air-conditioned room (<10ºC). The skin colour of individual fish was re-recorded after 6 and 22 h. The temperature of the chilled fish did not exceed 0ºC at any time during storage.

2.7 Market appreciation trial

A group of plate-size snapper were artificially darkened in a black, 9 kL outdoor tank for 4 months and reared under protocols similar to those described earlier. Snapper were then anaesthetized (as above), and their individual weight and skin colour measurements recorded (wet weight = 409 ± 83 g, L^* = 44.9 ± 4.8, a^* = 3.7 ± 0.9, b^* = 7.5 ± 1.3; mean ± SD, n = 40), before 20 fish were randomly allocated to either white or black cages (i.e. 40 fish in total; outdoor system). Six randomly selected snapper were harvested from the white or black cages 9 days after stocking. The fish were dip netted from cages and transferred directly to a saltwater ice slurry (-0.3ºC; 30 g L^{-1}). After 0.75 h, snapper were removed from the ice slurry and skin colour was quantified. The 12 fish were then finger packed into a polystyrene box under a protective layer of plastic and covered with ice. These fish were presented to experienced wholesalers at the Sydney Fish Market (SFM; Pyrmont, NSW, Australia) and the Newcastle Commercial Fishermen's Co-operative (NCFC; Wickham, NSW, Australia) for an independent market appraisal 18 h after harvest. At this time, the skin colour of these fish was remeasured. In addition, the skin colour of 12 wild-caught snapper presented for sale at the SFM was quantified for comparative purposes.

2.8 Statistical analyses

Before analyses of variance (ANOVA), data were assessed for homogeneity of variance using Cochran's test (Winer, 1971). If necessary, the Student-Newman-Keuls (SNK) test was used to

separate significant treatment means. All statistical tests were conducted using STATGRAPHICS PLUS, Version 4.1 (Manugistics, Rockville, MD, USA) with α set at 0.05.

Skin colour data from parts A and part B of the experiment were subjected to a two-factor ANOVA in order to study the interaction between the main effects (i.e. cage colour and light environment). For part A, the average *L**, *a** or *b** value of the five snapper in each experimental cage was used in statistical tests (i.e. cage as replicate). Owing to the repetitive sampling of replicate cages on day 2, 7 and 14, data collected at each time was analysed separately. Cochran's test indicated there were heterogeneous variances among treatments for *L**, *a** and *b** values in experimental data from part A. Investigation revealed one data point had a studentized residual >3.0. This outlier was related to one replicate cage that contained a moribund fish. Removal of this fish from the data set restored homogeneity of group variances in all further tests.

For part B, the average *L**, *a** or *b** value of the two snapper from each experiment cage was used in statistical tests (i.e. cage as replicate). Owing to the repetitive sampling of replicate cages 0.75, 6 and 22 h post-mortem, data collected at each time were analysed separately.

A two-factor ANOVA was used to examine the effect of cage colour (black or white) and time (0.75 h or 18 h; time considered as a fixed factor), on the post-mortem skin colour of snapper (i.e. fish = replicate). In addition, a one-factor ANOVA was used to compare the post-mortem skin colour of snapper conditioned in black (n = 6 individuals) or white cages (n = 6 individuals) from PSFC to the post-mortem skin colour of wild snapper on sale at the SFM (n = 12 individuals).

3. RESULTS

3.1 Effect of cage colour and light environment on skin colour – part A

The *L** skin colour value of live snapper was not significantly affected at any time by the light environments provided in this experiment or the interaction between the main effects ($P > 0.05$). However, the *L** values were significantly affected by cage colour and were higher (i.e. lighter) in snapper held in white cages compared with snapper held in black cages ($P < 0.05$; Table 1).

No interactions were detected between the main effects with respect to *a** skin colour values at any time ($P > 0.05$), but a weak interaction was detected between the main effects for values of *b**, but only on day 14 ($P = 0.034$; Table 1). Both main effects significantly affected *a** or *b** skin colour values at different times; however the magnitude of these changes was minor compared to changes in the value of *L**.

3.2 Effect of cage colour and light environment on post-mortem skin colour – part B

The *L**, *a** or *b** skin colour values of snapper measured 0.75, 6 or 22 h post-mortem were not significantly affected by light environment, or by the interaction between the light environment and cage colour ($P > 0.05$; Table 2). Irrespective of the time elapsed, the post-mortem value of *L** remained significantly higher (lighter) in snapper taken from white cages compared with those taken from black cages. In addition, despite a small decrease in value, the *L** skin values remained relatively stable over time, regardless of whether snapper were taken from white or black cages (Table 2).

Post-mortem changes in *a** and *b** skin colour values were more variable, and the values of both tended to decline over time. Snapper taken from black cages remained significantly redder (higher *a**) than snapper taken from white cages; however, there was no difference between the *b** skin colour values of snapper at any time (Table 2).

3.3 Market appreciation trial

Two-factor ANOVA indicated that the post-mortem *L**, *a** or *b** skin colour values of snapper were significantly affected by cage colour and time; however, there was no interaction between these factors for any skin colour variable ($P > 0.05$; Table 3).

The skin colour of wild snapper on display at the SFM was significantly lighter (higher *L**) and redder (higher *a**) than the skin colour of fish from PSFC ($P < 0.05$); however, according to *b** values, fish from black cages were more yellow than wild fish ($P < 0.05$, Table 3).

Based on skin colour alone, nine of the 11 buyers interviewed at the SFM and NCFC indicated that they preferred the cultured snapper conditioned in white cages while two buyers were not interested in either of the colour forms. The improved price differential for fish reared in white cages was between 10% and 50%, while some buyers indicated that they would only purchase the lighter fish from white cages and were entirely disinterested in the darker fish. Overall, all observers believed that the snapper we presented were not as acceptable as wild fish Because of their lack of pinkness; however, light-coloured fish were more acceptable than those harvested from black cages.

4. DISCUSSION

4.1 Effect of cage colour and light environment on skin colour

The transfer of pre-conditioned snapper to white cages significantly improved skin lightness (i.e. higher *L** values), irrespective of whether snapper were held in cages located outdoors in full sun or in the reduced light environment of our shade-house. This improvement was rapid and was sustained over a period of 14 days. Transferring dark-skinned snapper to white cages also significantly affected *a** and *b** skin colour values, but these changes were relatively minor and somewhat variable compared with the change in the magnitude of *L** values (Table 1). Incident light readings taken on cloudless days confirmed that PAR was substantially reduced within the shade-house and that this reduction was due predominantly to the transmission qualities of the materials covering this structure (Figure 1). These materials altered both the quanta (intensity) and spectral range (from UV to far red) of the light transmitted to the water surface and below, but these alterations of the spectrum did not influence the *L** skin colour of snapper in this study. Significant but relatively minor differences were detected in *a** and *b** skin colour values of snapper subjected to natural or reduced levels of sunlight at different times (Table 1), but the magnitude of these differences was of little practical importance.

While a reduced light environment did not affect the *L** skin colour value of snapper in this study, other studies have found that shading from direct sunlight reduced the dark appearance of *Pagrus* spp. (Matsui, *et al.* 1992; Booth, *et al.* 2004 [Chapter 4.1]). This difference may be a consequence of the greater degree of shading (i.e. reduction in intensity) or exclusion of specific wavelengths (Szisch, *et al.* 2002; Van der Salm, *et al.* 2004) provided by the cited authors (i.e. Matsui, *et al.* 1992; Booth *et al.* 2004 [Chapter 4.1]) compared with that provided in our experiment. As such, treatments that exclude the majority of natural light may reduce the visible cues that prompt *Pagrus* spp. like snapper to adapt cryptically to the colour of their immediate surroundings. We note that the cubic experiment cages used by Booth, *et al*. (2004 [Chapter 4.1]) in their experiments on *P. auratus* were black and excluded natural sunlight from the surface of cages as well as the walls.

The monochromatic colour of the experiment cages used in this study proved to be the overwhelming factor governing the skin lightness of snapper. A relationship between the colour of the background and the integument of sand flathead has also been reported (Douglas & Lanzing, 1981). Cage colour (i.e. black or white) appears to have directly influenced the neuro-endocrine system of snapper, which

has in turn influenced the physiological state of the melanophores contained in their skin. Presumably, snapper transferred to white cages have probably experienced a rapid aggregation of melanosomes within melanophores while the opposite has occurred in snapper subjected to black cages or black-lined tanks (Fujii & Oshima, 1986). Further studies are necessary to confirm this presumption.

4.2 Effect of cage colour and light environment on post-mortem skin colour

The marked difference between the L^* values of snapper held for 16 days in either black or white cages remained evident up to 22 h after they were killed in a saltwater ice slurry and stored under ice (Table 2). Despite the maintenance of this difference, a slight reduction in L^* value was observed in both colour groups, indicating that fish were becoming darker post-mortem. This effect was also observed in snapper killed and chilled for the market evaluation trial (Table 3). Although microscopic observations were not performed, a possible explanation for the initial fall in L^* values of snapper held in white cages from 69 to 66 (i.e. 6 h; Table 2) is the rapidity with which melanosomes dispersed within melanophores (Lin, Ushio, Ohshima, Yamanaka & Koizumi, 1998b). In addition, while not specifically observed in the present study, a reduction in the net amount of melanin and or the number of skin melanophores may have occurred in snapper held in white cages (i.e. morphological change; see Fujii, 1993). A similar effect has been reported by Van der Salm, *et al.* (2004), who observed a decrease in the number of scale melanophores in red porgy exposed to a white background.

The a^* and b^* skin colour values of chilled snapper also declined over time, with fish becoming less red and less yellow after the 22- or 18-h studies (Tables 2 and 3 respectively). A plausible explanation for the reduction in these skin colour values is that the short-term storage of snapper in direct contact with ice caused 'blanching' of the skin with a resultant loss of chromaticity (i.e. reduced a^* and b^* values). A reduction in chromaticity was also recorded for snapper used in the market evaluation trial; however, this reduction was not as severe as it was for the snapper stored in direct contact with ice. This suggests that the use of a barrier to prevent direct contact between the skin of chilled fish and ice, as currently used following commercial harvesting, would be beneficial in terms of improving the visual quality (chromaticity) of snapper reaching the market.

4.3 Market appreciation trial

Snapper held in white cages recorded 0.75 h post-mortem L^* skin colour values that were similar to those of wild-caught snapper presented for sale at the SFM (i.e. $L^* = 71$ vs $L^* = 72$; Table 3). This value was also higher than the lightest L^* value recorded for farmed snapper ($L^* \approx 63$) reared in heavily shaded, outdoor cages (Booth *et al.* 2004 [Chapter 4.1]). Unfortunately, by the time snapper were presented to wholesale buyers at the SFM approximately 18 h post-mortem, the L^* values of fish from white cages had declined significantly and were approximately eight units lower than wild-caught snapper (Table 3).

Despite the overall reduction in the skin lightness of both groups of snapper presented to wholesalers at the SFM and NCFC, most were complimentary about the appearance of snapper conditioned in white cages compared with those conditioned in black cages. These buyers estimated that a premium of between 10% and 50% could be expected for the lighter-coloured snapper we presented. However, most wholesalers were adamant that they would be reluctant to purchase dark- or light-skinned farmed snapper while the availability of wild-caught snapper was adequate. In addition, all buyers interviewed believed that if farmed snapper are to compete with the wild harvest, they must attain a more natural pigmentation, especially with respect to the red/pink colour of the skin.

Carotenoids such as astaxanthin have been used to enhance the pigmentation of *P. major* (Katayama, Shintani, Shimaya, Imai & Chichester, 1972; Tanaka, Katayama, Simpson & Chichester, 1976;

Nakazoe, Ishii, Kamimoto & Takeuchi, 1984; Ito, Kamata, Tanaka & Sameshima, 1986) and have been used to improve the pink/red pigmentation of snapper grown under farm conditions in Australia (Booth, *et al.* 2004 [Chapter 4.1]). However, for snapper farmed in sea cages under Australian conditions, the beneficial effects of astaxanthin are negated by the overwhelming display of melanophores that mask any underlying improvements in skin pigmentation. These problems may be overcome if, in addition to dietary supplementation with carotenoids like astaxanthin, 'white-cage technology' such as that tested here can be implemented on farm.

5. CONCLUSION

The results of our factorial study indicate that Australian snapper can rapidly alter the colour of their skin after being exposed to white backgrounds. However, reducing the intensity and altering the spectral profile of natural sunlight had no apparent effect on the L^* skin colour value. The rapid response to cage colour can be used to significantly lighten the skin of dark-coloured snapper before harvest procedures, which has the potential to improve the wholesale market price of farmed snapper by between 10 and 50%.

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TABLE 1
Skin colour values of snapper after 2, 7 and 14 days in black or white cages located in a natural or reduced sunlight environment [1].

	2 days			7 days			14 days [2]		
	*L**	*a**	*b**	*L**	*a**	*b**	*L**	*a**	*b**
Light environment									
Natural	55.7 ± 2.0	2.9 ± 0.2	7.9 ± 0.4 ^{x}	58.2 ± 3.2	3.1 ± 0.2 ^{x}	8.0 ± 0.2 ^{x}	55.9 ± 3.5	4.2 ± 0.2	8.1 ± 0.2 ^{x}
Reduced	57.4 ± 2.3	3.2 ± 0.2	8.7 ± 0.2 ^{y}	58.4 ± 3.6	3.7 ± 0.3 ^{y}	9.4 ± 0.4 ^{y}	54.2 ± 3.8	3.8 ± 0.2	9.0 ± 0.3 ^{y}
Cage colour									
White	61.8 ± 1.1 ^{b}	2.8 ± 0.2	8.8 ± 0.3 ^{b}	67.1 ± 0.6 ^{b}	2.9 ± 0.1 ^{a}	9.3 ± 0.4 ^{b}	64.5 ± 0.7 ^{b}	3.6 ± 0.2 ^{a}	9.0 ± 0.4 ^{b}
Black	53.4 ± 0.6 ^{a}	3.2 ± 0.2	8.3 ± 0.3 ^{a}	52.7 ± 0.8 ^{a}	3.8 ± 0.2 ^{b}	8.7 ± 0.3 ^{a}	48.5 ± 0.7 ^{a}	4.3 ± 0.2 ^{b}	8.4 ± 0.1 ^{a}

[1] Values represent the pooled level means ± SEM (n = 8 cages) of each factor at each sampling time. For each time, different superscript letters indicate a significant difference ($P < 0.05$; two-factor ANOVA, SNK) between skin colour variables within the levels of each factor (x, y: light environment; a, b: cage colour).
[2] According to two-factor ANOVA, there was a significant interaction ($P = 0.034$) between main effects for b^* at day 14.

TABLE 2
Post-mortem skin colour values of snapper sampled 0.75, 6 and 22 h after immersion in a salt-water ice slurry [1].

		0.75 h			6 h			22 h		
		*L**	*a**	*b**	*L**	*a**	*b**	*L**	*a**	*b**
Cage colour										
	White	69.0 ± 0.5 [b]	1.3 ± 0.3 [a]	8.1 ± 0.4	66.2 ± 0.6 [b]	1.1 ± 0.3 [a]	6.8 ± 0.3	66.4 ± 0.9 [b]	-0.4 ± 0.3	4.4 ± 0.4
	Black	47.2 ± 0.8 [a]	2.6 ± 0.1 [b]	7.7 ± 0.3	46.3 ± 0.5 [a]	2.4 ± 0.2 [b]	6.9 ± 0.3	44.7 ± 0.9 [a]	0.4 ± 0.2	4.1 ± 0.2

[1] Values represent the pooled level means ± SEM (n = 8 cages) of the fixed factor 'cage colour' at each sampling time. For each time, different superscript letters indicate a significant difference ($P < 0.05$; two-factor ANOVA, SNK) between the skin colour variables in each column

TABLE 3
Post-mortem skin colour values of chilled snapper from black or white cages after 0.75 and 18 h and comparisons with wild snapper.

	0.75 h			18 h		
	*L**	*a**	*b**	*L**	*a**	*b**
Cage colour[1]						
Black	$58.3 \pm 1.6^{a}{}_{y}$	4.7 ± 0.6^{b}	$10.6 \pm 0.5_{y}$	$54.8 \pm 1.6^{a}{}_{x}$	2.9 ± 0.5^{b}	$8.4 \pm 0.3_{x}$
White	$71.0 \pm 1.5^{b}{}_{y}$	2.5 ± 0.7^{a}	$9.6 \pm 0.5_{y}$	$64.8 \pm 1.2^{b}{}_{x}$	2.0 ± 0.5^{a}	$7.4 \pm 0.7_{x}$
Origin[2]						
Black – PSFC				54.8 ± 1.6^{a}	2.9 ± 0.5^{a}	8.4 ± 0.3^{b}
White – PSFC				64.8 ± 1.2^{b}	2.0 ± 0.5^{a}	7.4 ± 0.7^{ab}
Wild – SFM				72.4 ± 0.8^{c}	5.9 ± 0.5^{b}	6.2 ± 0.5^{a}

[1] Values represent mean ± SEM skin colour of $n = 6$ snapper from black or white cages at PSFC. Different superscript letters (a,b) between cage colours, or different subscript letters (x,y) between times indicate a significant difference ($P < 0.05$; two-factor ANOVA, SNK) between skin colour variables within the levels of each factor.

[2] Values represent mean ± SEM skin colour of snapper from the Port Stephens Fisheries Centre (PSFC; $n = 6$ fish per treatment) visually appraised by wholesale buyers at the Sydney Fish Market (SFM) and compared with wild-caught snapper (n = 12) presented for sale at SFM. Origins displaying the same letter in the superscript are not significantly different ($P > 0.05$; one-factor ANOVA, SNK).

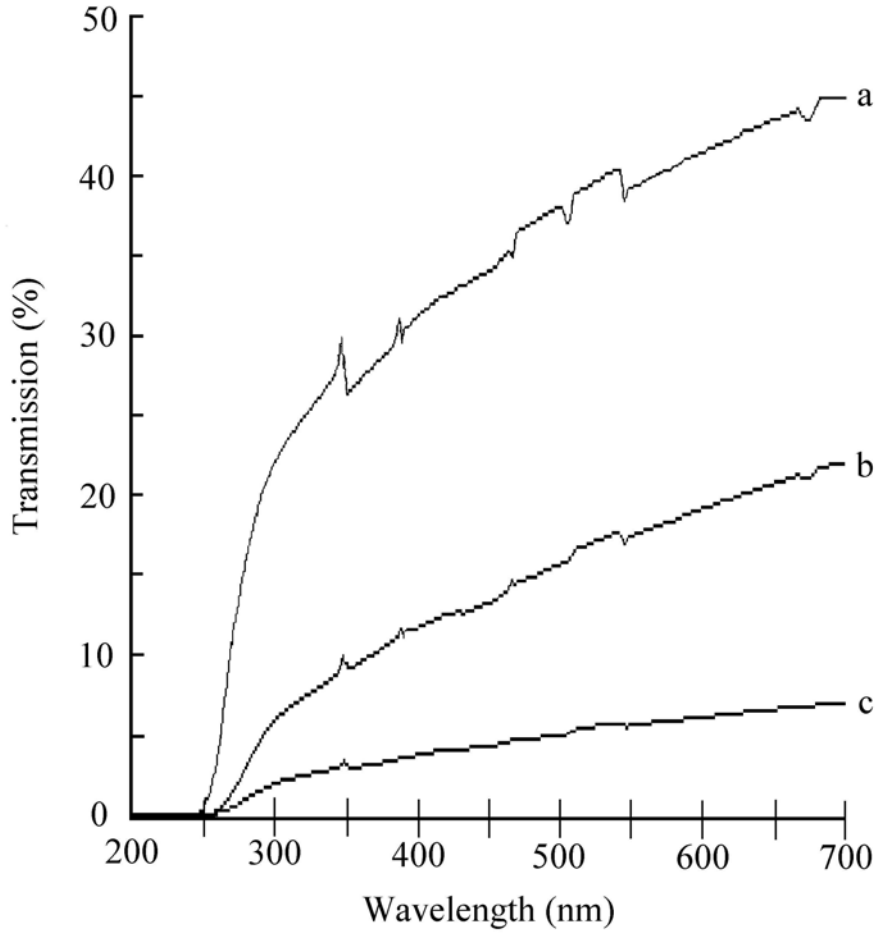

FIGURE 1
Spectral transmission qualities of the shade-house fabrics; (a) single layer of translucent plastic sheeting; (b) double layer of plastic sheeting; (c) double layer of plastic sheeting and single layer of shade-cloth (as fitted).

4.3 Effect of carotenoids and background colour on the skin pigmentation of Australian snapper *Pagrus auratus* (Bloch & Schneider, 1801)

Ben J. Doolan[1,2], Geoff L. Allan[2], Mark A. Booth[2]and Paul L. Jones[1]

[1] *School of Life and Environmental Sciences, Deakin University, Warrnambool, Victoria 3280, Australia*
[2] *NSW Department of Primary Industries, Port Stephens Fisheries Centre and Aquafin CRC, Private Bag 1, Nelson Bay NSW 2315*

ABSTRACT

Three two-factor experiments were conducted to determine the effects of background colour and synthetic carotenoids on the skin colour of Australian snapper *Pagrus auratus*. Initially we evaluated the effects on skin colour of supplementing diets for 50 days with 60 mg kg^{-1} of either astaxanthin (LP; Lucantin® Pink, BASF), canthaxanthin (LR; Lucantin® Red), apocarotenoic acid ethyl ester (LY; Lucantin® Yellow), selected combinations of the above, or no carotenoids, and holding snapper (mean weight = 88 g) in either white or black cages. In a second experiment, all snapper (mean weight = 142 g) from Experiment 1 were transferred from black to white, or white to white cages to measure short-term effects of cage colour on skin *L**, *a**, *b** colour values. Skin colour was measured after 7 and 14 days, and total carotenoid concentrations determined after 14 days.

Cage colour was the dominant factor affecting the skin lightness of snapper with fish from white cages much lighter than fish from black cages. Diets containing astaxanthin conferred greatest skin pigmentation and there were no differences in redness (*a**) and yellowness (*b**) values between snapper fed 30 or 60 mg astaxanthin kg^{-1}. Snapper fed astaxanthin in white cages displayed greater skin yellowness than those in black cages. Transferring snapper from black to white cages increased skin lightness but was not as effective as growing snapper in white cages for the entire duration. Snapper fed astaxanthin diets and transferred from black to white cages were less yellow than those transferred from white to white cages despite the improvement in skin lightness (*L**), and the total carotenoid concentration of the skin of fish fed astaxanthin diets was lower in white cages. Diets containing canthaxanthin led to a low level of deposition in the skin while apocarotenoic acid ethyl ester did not alter total skin carotenoid content or skin colour values in snapper.

In a third experiment, we examined the effects of dietary astaxanthin (diets had 60 mg astaxanthin kg^{-1} or no added carotenoids), and cage colour (black, white, red or blue) on skin colour of snapper (mean weight = 88 g) after 50 days. Snapper fed the astaxanthin diet were more yellow when held in red or white cages compared with fish held in black or blue cages despite similar feed intake and growth. The skin lightness (*L** values) was correlated with cage *L** values, with the lightest fish obtained from white cages. The results of this study suggest that snapper should be fed 30 mg astaxanthin kg^{-1} in white cages for 50 days to increase lightness and the red colouration prized in Australian markets.

1. INTRODUCTION

Interest in commercial culture of Australian snapper *Pagrus auratus* and the recognition that snapper cultured in sea cages have a much darker skin without the distinct pink or red pigment found in wild snapper has prompted investigation into methods of altering skin colour to optimise market value. Similarly, vast differences in colour between cultured and wild specimens of the closely related red sea bream *P. major* (Paulin, 1990; Tabata & Taniguchi, 2000) have been observed since the 1960s (Katayama, Ikeda & Harada, 1965) and more recently in red porgy *P. pagrus* (Van der Salm, Martinez, Flik & Wendelaar Bonga, 2004; Kalinowski, Robaina,

Fernández-Palacios, Schuchardt & Izquierdo, 2005; Van der Salm, Pavlidis, Flik &Wendelaar Bonga, 2006). While background adaptation to white cages can drastically reduce the dark colouration of snapper (Doolan, Booth, Jones & Allan, 2007 [Chapter 4.2]), limited research has been published on the benefits of supplementing feeds with different carotenoids (Booth, Warner-Smith, Allan & Glencross, 2004 [Chapter 4.1]), despite their importance as ingredients used to enhance pigmentation in cultured fish to increase acceptance in the marketplace (Nickell & Springate, 2001).

Animals are unable to synthesise carotenoids *de novo* (see review by Britton, Liaaen-Jensen & Pfander, 1998) and snapper, like many marine teleosts, require a dietary source of carotenoids that can be utilized for skin pigmentation (Simpson & Kamata, 1979). In contrast to the natural diets of wild snapper, carotenoids are absent in standard commercial feeds for farmed snapper in Australia. Astaxanthin (3,3′-dihydroxy-4,4′-diketo-β,β-carotene) is the most commonly occurring carotenoid in marine animals (Torrissen & Christiansen, 1995) and is widely used in aquaculture feeds to enhance flesh pigmentation, especially for salmonids (Torrissen, 1995; Forsberg & Guttormsen, 2006). Furthermore, astaxanthin is considered to be the main carotenoid responsible for pink skin pigmentation in *P. major* (Katayama, *et al.* 1965; Tanaka, Katayama, Simpson & Chichester, 1976), especially when supplied as mono- or di-esters in feeds (Nakazoe, Ishii, Kamimoto & Takeuchi, 1984; Ito, Kamata, Tanaka & Sameshima, 1986; Ha, Kang, Kim, Choi & Ryu, 1993). It has also been suggested that a combination of the xanthophylls canthaxanthin (4,4′-diketo-β,β-carotene) and apocarotenoic acid ethyl ester (β-apo-8′-carotenoic acid ethyl ester) can produce pink skin colour in *P. major* (Sanders, 1975). Canthaxanthin is frequently used in the Atlantic salmon *Salmo salar* industry to deeply enhance the red colouration of the flesh (Baker, 2002) and is preferred by some growers Because of its cost effectiveness over the use of astaxanthin (Buttle, Crampton and Williams, 2001). Apocarotenoic acid ethyl ester, on the other hand, is more typically used in the poultry industry for colouring egg yolks and broilers (Paust, 1991; Ponsano, Pinto, Garcia-Neto & Lacava, 2004).

While the supplementation of carotenoids in diets may increase the pinkness of snapper, the beneficial effects may be masked by the overlying darkness exhibited by the melanophores of fish under sea cage culture conditions, both when alive (Booth *et al.* 2004 [Chapter 4.1]), and after slaughter (Hatae, 1990; Matsui, Tanabe, Furuichi, Yoshimatsu and Kitjama, 1992; Kolios, Kiritsis & Katribusas, 1997; Lin, Ushio, Ohshima, Yamanaka & Koizumi, 1998a, b). Skin colour adaptation to background colours has been studied in sparids (Van der Salm, Martinez, Flik & Wendalaar Bonga, 2004; Van der Salm, *et al.* 2006; Doolan, *et al.* 2007 [Chapter 4.2]) primarily to improve skin lightness. While it is possible to improve skin colour from dark to light by transferring fish to white surroundings for a short period (eg. 2 – 14 days) before harvest, the combined effects of background colour and dietary carotenoids have not been studied. In addition, it is unknown whether dark pigmented snapper supplemented with carotenoids will lighten and retain the pink colouration after transfer to white surroundings.

To explore these issues, three experiments were carried out to determine the effects on snapper skin colour of (1) feeding snapper diets supplemented with different commercially available carotenoids (singly or in combinations) in black or white cages, (2) transferring snapper fed diets supplemented with different carotenoids to either black or white cages, and (3) astaxanthin supplementation on fish held in cages of different colour.

2. MATERIALS AND METHODS

2.1 Experimental design and colour measurement

All experiments were balanced two-fixed-factor analysis of variance (ANOVA) designs. Experiment 1 evaluated the effects of background cage colour (2 levels; black or white) and dietary carotenoid type [6 levels; 60 mg astaxanthin kg^{-1} (LP60), 60 mg canthaxanthin kg^{-1} (LR60), 60 mg apocarotenoic acid ethyl ester kg^{-1} (LY60), 30 mg astaxanthin and 30 mg apocarotenoic ester kg^{-1}

(LP30-LY30), 30 mg canthaxanthin and 30 mg apocarotenoic acid ethyl ester kg^{-1} (LR30-LY30), or a control diet with no carotenoids (C0])] on snapper skin colour after 50 days. Experiment 2 examined the effects of transferring snapper fed on the abovementioned diets (6 levels; described previously) from black to white cages (three levels; black to white, black to black control, or white to white control) for 7 and 14 days. Experiment 3 investigated the effects of background cage colour (four levels; black, white, red or blue) and astaxanthin [two levels; 60 mg kg^{-1} (LP60) or 0 mg kg^{-1} ([C0)] for 50 days. Treatments comprised of five replicate cages each containing five fish in all experiments.

Skin and cage colours for all experiments were quantified according to the CIE *L*a*b** colour space whereby *L** represents lightness (black 0 to white 100), *a** represents green (-60) to red (+60), and *b** represents blue (-60) to yellow (+60). Skin colour was measured using a handheld chroma-meter (Minolta CR-10; Minolta, UK: Selby Biolab, Gladesville, NSW, Australia) from three positions along the left flank of each fish and averaged to provide a representative *L**, *a** and *b** value for each fish as described by Booth *et al.* (2004 [Chapter 4.1]).

2.3 Diets

Experimental diets were produced by re-milling a commercial barramundi *Lates calcarifer* diet (50% protein, 12% fat; Barra Grower 18MJ; Ridley Agriproducts, Narangba, Qld, Australia) at the Port Stephens Fisheries Centre (PSFC). The commercial diet was ground through a hammer mill (Raymond Laboratory Mill; Transfield Technologies, Rydalmere, Australia) fitted with a 1.5 mm screen. Diets were thoroughly mixed (Hobart Mixer; Hobart Corporation, Troy, OH, USA) with the inclusion of the required quantity of carotenoid(s) and 0.1% Vitamin C (L-ascorbic acid phosphate; CCD Animal Health, Girraween, NSW, Australia) before the addition of approximately 600 mL distilled water kg^{-1}. The basal diet (C0) devoid of carotenoids was treated in a similar manner to all other diets. The mixture was pelleted through a meat mincer fitted with a 4 mm pellet die (Barnco Australia Proprietary, Leichhardt, NSW, Australia). Diets were placed in a convection drier at <40^{o}C for approximately 6 h until moisture contents were <10% to produce a dry, slow sinking pellet. All diets were stored in the dark at –20^{o}C after pelleting. Treatments in each experiment were randomly assigned with diets containing a total of 60 mg carotenoid kg^{-1} or a control diet containing no added carotenoids. All unesterified carotenoid preparations [Lucantin® Pink (LP) Lucantin® Red (LR) and Lucantin® Yellow (LY); 10% formulations] were kindly donated by BASF, Ludwigshafen, Germany.

2.4 Experimental facilities

All experiments were conducted in a saltwater recirculating system located inside a hot-house. The system consisted of 11 circular fibreglass tanks (3.4 m diameter; 1.1 m deep; 10,000 L) as described in Doolan *et al.* (2007 [Chapter 4.2]). Eight circular cages were held within each tank (total = 85 cages). Cages (600 mm diameter; 700 mm depth; volume = 200 L) were assembled from 10 mm oyster mesh and lined with white (*L** = 89.1, *a** = -1.0, *b** = 0.4), black (*L** = 25.4, *a** = -1.0, *b** = 0.3), blue (*L** = 47.8, *a** = -14.1, *b** = -35.9) or red (*L** = 36.1, *a** = 44.9, *b** = 16.0) 420 μm PVC plastic consisting of a woven polyester base fabric (Ricky Richards Pty. Ltd., Homebush, NSW, Australia). Cage bases were constructed from 4 mm oyster mesh. Recirculated water flowed through 15 mm polyethylene pipes fitted around the circumference of each tank with individual 15 mm inlets delivering water at ~2.5 L min^{-1} into each of the eight cages. All tanks were aerated using 2 x 100 mm air stone diffusers and water temperatures were maintained via the installation of 1 kW immersion heaters. 15 μm system cartridge filters were exchanged daily and tanks were vacuum siphoned every second day to ensure the removal of accumulated solids. Water quality was measured daily with a Horiba U-10 Water Quality Analyser (Horiba, Kyoto, Japan) and total ammonium-nitrogen levels were determined by colourimetric means (Aquamerck; Model 1.08024; Merck, Darmstadt, Germany). Water quality variables in Experiments 1 and 3 combined, and Experiment 2, respectively, were dissolved oxygen >6.41, >7.1 mg L^{-1}, pH 7.71–8.39, 7.85–

8.27, salinity 28.5–33.3, 30.6–32.2 g L^{-1}, temperature 17.8–23.7, 18.0–21.9 ^{o}C, and total ammonia <0.4, <0.3 mg L^{-1}.

2.5 Fish and feeding

Australian snapper were produced at PSFC, raised in pale blue 10,000 L tanks (L^* = 74.6, a^* = -12.9, b^* = 3.5) in a recirculating saltwater system and fed a commercial diet (Barra Grower 18MJ; Ridley Agriproducts) twice daily before stocking in experiment cages. Upon stocking, fish in tanks were anaesthetized with 10 mg L^{-1} ethyl-*p*-aminobenzoate *in situ* and randomly transferred to an oxygenated translucent plastic tub (200 L) for further sedation (20–25 mg L^{-1} ethyl-*p*-aminobenzoate). Individual colour measurements and weights of fish were obtained before systematic distribution throughout the 200 L experiment cages. Fish were handfed to apparent satiation twice daily at 09:00 and 15:00 hours (Australian Eastern Standard Time) and starved for one day before subsequent colour and weight collections. At the completion of the experiments, the 200 L tub described above was submerged and placed beneath each cage and fish sedated as described previously.

2.6 Experiment 1: Effect of carotenoids and monochromatic cage colour on skin pigmentation of snapper

300 snapper (89 ± 13 g; mean ± SD) held in a pale blue 10,000 L tank were anaesthetised, measured for colour, weighed and stocked in randomly positioned black or white 200 L cages. Fish were fed twice per day on one of six diets (LR60, LP60, LY60, LR30-LY30, LP30-LY30 or C0) and daily feed intake was recorded. After 50 days, snapper were removed under sedation and the skin colour and weight of all individuals were measured.

2.7 Experiment 2: Effect of carotenoids and short-term adaptation to cage colour on skin pigmentation of snapper

Upon completion of Experiment 1, snapper (142 ± 18 g; mean ± SD) were returned to their original random cage positions in their initial groups of five fish. Snapper held in white cages of Experiment 1 were returned to their original white cages (white) and snapper previously held in black cages were transferred to white cages (black-white). An additional five black cages each containing five fish and allocated the basal (C0) diet were grown concurrently with Experiment 1 and were returned to black cages to function as black cage controls for Experiment 2. Diets and feeding resumed as for Experiment 1. After 7 days, cages of snapper were individually anaesthetised, measured for colour and all fish returned to the cages. After 14 days, the process was repeated and two representative snapper from each cage killed with a lethal dose of ethyl-*p*-aminobenzoate (100 mg L^{-1}), and the skin from the left flank was immediately removed with a sharp knife and stored at -80^{o}C for total carotenoid determination. Before carotenoid analysis, the chromatophores of skin samples were observed microscopically.

2.8 Experiment 3: Effect of astaxanthin and cage colour on skin pigmentation of snapper

200 snapper (88 ± 12 g; mean ± SD) were removed from their pale blue holding tank, measured for colour, weighed and stocked into black, white, blue or red 200 L cages. Cages were randomly positioned among the cages of Experiment 1. Each cage colour was randomly assigned one of two diets (LP60 or C0) and fed to apparent satiation twice daily. The experiment was terminated after 50 days when weight and colour measurements were obtained.

2.9 Carotenoid extraction and analysis

Carotenoid extraction from skin samples of Experiment 2 was carried out according to Barua, *et al.* (1993). Samples were slowly thawed from –80^{o}C and snapper skin (0.5 – 0.9 g) was homogenized with scissors, weighed, further ground in 10 mL ethyl acetate:ethanol (1:1 v/v) and left to extract

for 15 min. The supernatant was removed following centrifugation at 6000 rpm (4000 *g*) for 2 min and the pellet extracted with 5 mL ethyl acetate, and then 10 mL hexane until the solution was colourless. The three supernatants were combined and evaporated to dryness under vacuum, resuspended in 2 mL hexane containing 0.02% butylated hydroxytoluene (BHT) (w/v) (Torrissen 1995) and centrifuged [15,000 rpm (5000 *g*) 4 min]. Total carotenoid concentration was quantified spectrophotometrically in hexane (containing 0.02% BHT) at 470 nm using $E_{1\%1cm} = 2100$.

2.10 Statistical analyses

Data were assessed for homogeneity of variance using Cochran's test and $\log_{10}$ transformed to homogenize variances when required. In each experiment, two-factor ANOVA was conducted (where cages were treated as replicates), using STATGRAPHICS Plus V4.1 (Manugistics, Rockville, MD, USA). The Student-Newman-Keuls (SNK) test was performed to separate treatment means where significant differences ($P < 0.05$) occurred. The procedure was carried out after each sampling time to identify treatment effects on *L**, *a**, and *b** colour values, total carotenoid concentration and growth indices. Correlation co-efficients were used to portray associations between *L**, *a** and *b** skin colour values and total carotenoid concentrations at 64 days in Experiment 2, and non-linear regression analysis was used to investigate relationships between skin colour and cage colour values in Experiment 3.

3. RESULTS

3.1 Experiment 1

Cage colour strongly influenced skin lightness (*L**) with significantly darker fish observed from black cages (Table 1). Skin redness (*a**) was more intense in fish from black cages and yellowness (*b**) was significantly greater in fish held in white than black cages. A greater elevation in *b** values for fish held in white cages and fed the LP60 and LP30-LY30 (astaxanthin) diets was responsible for the cage colour effect and observed interaction between cage colour and diet (Table 1). In contrast, *b** values of black cage LP60 and LP30-LY30 fish did not differ from that of the LR60 and LR30-LY30 (canthaxanthin) supplemented fish.

The addition of carotenoids to diets did not alter skin lightness ($P > 0.05$); however, it greatly affected *a** and *b** values (Table 1). Astaxanthin, at 60 mg kg^{-1} (LP60), and at 30 mg kg^{-1} in combination with apocarotenoic acid ethyl ester (LP30-LY30), produced the greatest levels of skin redness in fish from both black and white cages, and yellowness in the skin of white cage fish. Canthaxanthin (LR60 and LR30-LY30) also produced an elevation in skin redness values above basal levels but not to the level attained when fish were fed astaxanthin. LR60 and LR30-LY30 diets increased *b** values to the same level as LP60 and LP30-LY30 diets in black cage fish but not to the level of fish fed LP60 and LP30-LY30 in white cages. There were no differences in *L**, *a** or *b** values between snapper fed astaxanthin supplied at 60 mg kg^{-1} (LP60) or 30 mg kg^{-1} (LP30-LY30). LY60 caused no change in any of the colour components measured.

Diet had no significant effect on growth as indicated by feed conversion ratios (FCR), feed consumption or weight gain (Table 1). Cage colour did not affect FCR; however feed consumption and weight gain were significantly higher in fish held in black cages than white cages.

3.2 Experiment 2

Transferring snapper from black to white cages for 7 or 14 days produced much lighter skin (higher *L**) but not to the same extent as fish grown in white cages throughout the entire feeding period (Table 2). Control snapper fed the basal diet (C0) from black and white cages remained significantly discrete ($P < 0.05$) in skin lightness, each with negligible change during the 14 days of transfer. Observations of the skin revealed lower numbers of dermal melanophores in fish held long-term in white cages. Skin redness (*a**) after 7 and 14 days was not affected by cage colour (*P*

> 0.05) and there was no interaction between diet or cage colour ($P > 0.05$). Skin yellowness *(b**) after 7 and 14 days was affected by both diet and cage colour and there was a significant interaction (Table 2) Because of the similar effects of diet described in Experiment 1.

Carotenoids supplied in the diet led to higher *a** and *b** values and significantly darker (lower *L**) snapper after 7 days of transfer, most notably in the LP treatments (Table 2). There was no significant effect of diet on skin lightness after 14 days ($P = 0.057$). *a** values were significantly elevated in fish fed LP60 and LP30-LY30 diets at both times after transfer despite lower values at day 7 than day 14. *b** values of snapper were greatly elevated when fed LP diets in white cages at days 7 and 14 but not in those transferred from black to white cages.

A significant interaction between cage colour and diet occurred in the total carotenoid concentration of the skin, driven by higher levels in fish fed LP60 in black-white cages than fish held in white cages (Table 2). Total carotenoid concentration of snapper skin was highest in fish given the LP diets. LR diets increased total carotenoids in the skin of fish from the black-white treatments, while the levels in fish fed the LY60 diet did not alter from fish fed the basal diet (C0).

Because of interactions in total carotenoid concentration between diets and cage colour treatments, correlations between the total carotenoid concentration and colour values of fish from combined treatments suggested weak relationships. Improved estimates of skin *a** values were obtained by examining cage colour treatments separately, with fish from white cages *a** = 3.35 Ln (total carotenoid concentration) - 2.9; $R = 0.61$, and fish from black-white cages *a** = 2.69 Ln (total carotenoid concentration) - 2.2; $R = 0.69$.

3.3 Experiment 3

A significant relationship existed between skin *L** and cage *L** (skin *L** = 9.88 Ln (cage *L**) + 25.8; $P < 0.0001$, $R^2 = 0.57$) increasing in magnitude from black, to red and blue, to white cages (Table 3). Diet did not affect skin lightness (*L**) but strongly affected *a** and *b** values, with higher skin redness (*a**) and yellowness (*b**) observed after feeding LP60 compared with the basal diet (C0). *a** values of snapper did not differ between cage colours, while *b** values were higher in white and red cages when fish were fed LP60 (Table 3). This effect created a more yellow hue in fish fed LP60 diets in red and white cages than the more red hue observed in fish from blue and black cages. With regard to hue, fish fed the basal diet (C0) were more yellow than the LP treatments, especially in white cages (Table 3). However, the intensity of colour (chroma) of these fish was much lower, easily altering the hue angle by minor changes in magnitude; therefore, hue data were not presented in this study.

As for Experiment 1, FCR, daily feed consumption and weight gain were not affected by diet (Table 3). FCR was not affected by cage colour; however, feed consumption and subsequent weight gain was lowest in white cages.

4. DISCUSSION

Of the carotenoids tested in the present study with snapper, astaxanthin provided greatest skin pigmentation as indicated by total carotenoid concentration and colour values. Total carotenoids and *a** and *b** values of the skin increased when fish were fed diets supplemented with canthaxanthin but did not attain the intensity of fish fed astaxanthin. This supports the findings of Kalinowski, Kalinowski, Robaina, Fernandez-Palacios, Schuchardt and Izquierdo (2005) with *P. pagrus*. Apocarotenoic acid ethyl ester did not alter skin colour and was most likely excreted unmodified. These data are in accordance with Katayama, *et al.* (1965) and Tanaka, *et al.* (1976) who reported that astaxanthin is the most commonly occurring carotenoid in wild *P. major*. Tanaka, *et al.* (1976), suggested that *P. major* are unable to convert other carotenoids (such as canthaxanthin and zeaxanthin) to astaxanthin and as such belong to 'Group II' of a classification

devised by Tanaka (1978) based on the ability of animals to biosynthesise carotenoids to astaxanthin. Fish in this group must obtain astaxanthin from the diet for deposition in the skin.

Carotenoids occur in the skin of most fish as esters (Schiedt, 1998), and predominantly as diesters in the skin of *P. major* (Kayama, Nakagawa, Yamada & Murakami, 1973; Ha, *et al.* 1993). While esterified astaxanthin is reportedly more efficiently utilized in the skin of *P. major* (Nakazoe, *et al.* 1984; Ito, *et al.* 1986; Ha, *et al.* 1993), unesterified astaxanthin effectively enhanced skin pinkness in this study. An astaxanthin inclusion rate of 30 mg kg^{-1} was as effective as 60 mg kg^{-1} at producing pink skin (displayed by higher *a** and *b** values) over the 50-day period of Experiment 1 and may indicate a saturation level in astaxanthin deposition was attained. A saturation level for astaxanthin has been observed with deposition in the flesh of salmonids (Storebakken, Foss, Schiedt, Austreng, Liaaen-Jensen & Manz 1987; Choubert & Storebakken, 1989; Bjerkeng, Storebakken & Liaaen-Jensen, 1990; Storebakken & No, 1992; Torrissen, Christiansen, Struksnaes & Estermann, 1995) whereby carotenoid retention efficiency in the flesh decreases with higher inclusion levels in the diet. Therefore, it is important to achieve a high ratio of astaxanthin deposition to astaxanthin in the diet (Torrissen, 1985). Booth, *et al.* (2004 [Chapter 4.1]) suggested that skin pigmentation of snapper from unesterified astaxanthin was more evident at 72 mg kg^{-1} than 36 mg kg^{-1} after 3 weeks, but a possible biological saturation occurred beyond 6 weeks when there was no apparent difference between the two concentrations. These authors also reported that there was no difference in pigmentation between esterified (NatuRose™, Ctabitecg /ciroiratuibm Kaihia-Kona, HI, USA) and unesterified (Carophyll Pink™, Hoffman-La Roche, Basel, Switzerland) astaxanthin (60 mg kg^{-1}) over a 116-day period. Owing to the high cost of astaxanthin, further dose response studies are required with snapper to elucidate the optimal concentrations and duration of application with astaxanthin.

The supplementation of dietary carotenoids did not promote beneficial effects on growth, similar to recent findings on the related *P. pagrus* (Cejas, Almansa, Tejera, Jerez, Bolannos & Lorenzo, 2003; Kalinowski, *et al.* 2005) and *Sparus aurata* (Gomes, Dias, Silva, Valente, Empis, Gouveia, Bown and Young, 2002; Gouveia, Choubert, Pereira, Santinha, Empis & Gomes, 2002). However, a white background colour led to lower feed consumption and subsequent reduced weight gain in comparison with fish held in black, red or blue (i.e. darker) cages. This finding may indicate that fish in white cages were stressed, as reported by Rotllant, Tort, Montero, Pavlidis, Martinez, Wendelaar Bonga & Balm (2003) in *P. pagrus* on the basis of elevated plasma cortisol, glucose and α-melanophore stimulating hormone (α-MSH) when held in white rather than black tanks. Snapper grown in black cages (i.e. with greater feed consumption and weight gain) and fed astaxanthin (LP60) displayed a higher concentration of total carotenoids than snapper from white cages. This does not conform to a plateau in astaxanthin deposition as described above unless cage colour-dependent astaxanthin saturation levels occur in snapper as a form of cryptic colouration.

Our results clearly support previous research by Doolan, *et al.* (2007 [Chapter 4.2]) in that the colour of the culture environment has a strong effect on the level of lightness of the skin. A strong correlation was evident between cage lightness and skin lightness as previously reported with *P. pagrus* subjected to background colours of white, black and red (Van der Salm, *et al.* 2004), and in sand flathead *Platycephalus arenarius* exposed to different shades of background (Douglas & Lanzing, 1981). Snapper devoid of carotenoids were only capable of modifying skin colour by monochromatic changes, altering skin lightness through the expression of melanophores. However, once astaxanthin was utilized, snapper were capable of additional chromatic changes, likely via erythrosome dispersion within skin erythrophores. In particular, snapper held in white and red cages displayed greater skin yellowness than fish in black or blue cages, suggesting that snapper are not only capable of adapting skin pigmentation to the monochromatic shade but also to the hue of the surroundings when astaxanthin is present in the skin from the diet. The abnormal deep golden colour (indicated by elevations in *a** and *b**, and reduction in *L**) displayed by snapper fed astaxanthin in red cages was of particular interest and had not been observed previously in the species.

Although not as apparent as the dominant effect of cage colour, the skin of snapper fed astaxanthin was darker as reported by Pavlidis, Papandroulakis and Divanach (2006) in dorsal skin of *P. pagrus*. As such, some skin lightness is sacrificed by intensifying skin colour with astaxanthin.

Transferring snapper from black to white cages for up to 14 days greatly improved skin lightness but not to the level attained when fish were held for the entire culture period in white cages. The differences observed were Because of greater numbers of dermal melanophores in the previously black adapted fish. Physiological changes through an aggregation of melanosomes were responsible for the observed improvement in skin lightness of snapper transferred from black to white cages; however, longer exposure to white surroundings is likely required to induce a morphological reduction in the number of melanophores and subsequent greater improvements in skin lightness. In addition, the red appearance of snapper reduced after 7 days of transfer from black to white cages in Experiment 2. Similarly, snapper held long-term in white cages during Experiment 1 lost skin redness (a^*) after transfer to white cages in Experiment 2, particularly during the first 7 days. The reasons for these observations are unclear; however, they display the physiological chromatic changes that snapper can manifest. As such, attempting to relate the carotenoid concentration of snapper by measuring a^* and b^* values of the skin may be unreliable.

High concentrations of astaxanthin in the skin do not necessarily equate to high a^* and b^* values as observed in snapper fed astaxanthin (LP) diets and transferred from black to white cages. Astaxanthin only elevated b^* (yellowness) values when fish displayed a high level of lightness when held only in white cages throughout the experiments, or as a form of background adaptation when fish were held in red cages. A similar result was presented by Booth, *et al.* (2004 [Chapter 4.1]) who found that astaxanthin did not affect b^* values in their snapper that displayed lower skin lightness (L^* = ~60). Clearer relationships have been inferred accurately from salmonid flesh (Smith, Hardy & Torrissen 1992; Christiansen, Sruksnaes, Estermann & Torrissen, 1995) in which flesh colour appears to be solely based on carotenoid concentration, or from snapper when other environmental conditions (i.e. cage colour) have remained constant (Booth, *et al.* 2004 [Chapter 4.1]) and have not initiated a physiological colour change to the extent caused by cage colour in this trial. In the present study, correlations between total carotenoid concentration and a^* and b^* values of fish within cage colours provided improved approximations; however, they did not compare to the accuracy (R^2 = >80) reported by Booth, *et al.* (2004 [Chapter 4.1]) in snapper.

In conclusion, this study indicates that complex chromatic changes occur in the skin of cultured snapper arising from interactions between cage colour and carotenoids in the diet. Cage colour was the dominant factor causing skin darkness in snapper and unesterified astaxanthin was the main carotenoid responsible for enhancing the visible pinkness. 30 mg astaxanthin kg^{-1} proved effective in improving a^* and b^* values after 50 days and total carotenoid concentration when quantified after 64 days. We recommend producing snapper in white enclosures; however, as this may not be possible with production in sea cages, it may be pertinent to transfer snapper to white enclosures for short periods (such as the 14 days in this study) before sale. Further morphological changes may take longer, although vast improvements in skin lightness were made during this period.

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TABLE 1 Results of ANOVA (*P* values) and *L**, *a**, *b** skin colour values, feed conversion ratios (FCR), feed consumption (% biomass day^{-1}) and % weight gain of snapper held in different cage colours and fed different carotenoids for 50 days [1,2]. (Experiment 1)

Variable	*Cage colour*	*Diet*	*Cage colour* x *Diet*
*L** [3]	$P < 0.0001$	ns	ns
*a**	$P < 0.01$	$P < 0.0001$	ns
*b**	$P < 0.0001$	$P < 0.0001$	$P < 0.001$
FCR [3]	ns	ns	ns
% biomass day^{-1}	$P < 0.0001$	ns	ns
% weight gain	$P < 0.05$	ns	ns

Cage colour	*Diet*	*L** [3]	*a**	*b**	FCR [3]	% biomass day^{-1}	% weight gain
White	C0	71.3 ± 1.9^{y}	$2.9 \pm 0.8^{a\,x}$	8.3 ± 0.4^{a}	1.64 ± 0.05	1.49 ± 0.05^{x}	60.3 ± 1.4^{x}
	LR60	70.8 ± 2.3^{y}	$4.1 \pm 0.3^{ab\,x}$	11.7 ± 0.5^{b}	1.55 ± 0.07	1.42 ± 0.04^{x}	62.0 ± 3.1^{x}
	LP60	68.4 ± 2.0^{y}	$8.0 \pm 0.4^{c\,x}$	$13.7 \pm 0.2^{c\,y}$	1.77 ± 0.13	1.45 ± 0.04^{x}	54.2 ± 4.4^{x}
	LY60	70.6 ± 1.0^{y}	$4.2 \pm 0.3^{ab\,x}$	8.6 ± 0.5^{a}	1.57 ± 0.10	1.42 ± 0.05^{x}	61.2 ± 4.0^{x}
	LR30-LY30	71.6 ± 0.7^{y}	$4.8 \pm 0.6^{b\,x}$	11.4 ± 0.5^{b}	1.69 ± 0.05	1.44 ± 0.03^{x}	55.9 ± 1.5^{x}
	LP30-LY30	69.4 ± 1.0^{y}	$8.1 \pm 0.3^{c\,x}$	$13.2 \pm 0.5^{c\,y}$	1.69 ± 0.05	1.49 ± 0.02^{x}	58.7 ± 2.8^{x}
Black	C0	55.3 ± 1.1^{x}	$4.5 \pm 0.6^{a\,y}$	8.4 ± 0.5^{a}	1.59 ± 0.08	1.59 ± 0.04^{y}	69.6 ± 5.0^{y}
	LR60	57.4 ± 1.4^{x}	$5.9 \pm 0.6^{ab\,y}$	11.1 ± 0.2^{b}	1.53 ± 0.03	1.46 ± 0.07^{y}	64.2 ± 3.1^{y}
	LP60	57.9 ± 2.6^{x}	$8.9 \pm 0.6^{c\,y}$	$10.8 \pm 0.4^{b\,x}$	1.63 ± 0.11	1.63 ± 0.03^{y}	68.8 ± 4.2^{y}
	LY60	58.7 ± 2.1^{x}	$5.0 \pm 0.4^{ab\,y}$	8.2 ± 0.4^{a}	1.65 ± 0.04	1.56 ± 0.03^{y}	63.8 ± 2.1^{y}
	LR30-LY30	58.5 ± 1.7^{x}	$5.8 \pm 0.4^{b\,y}$	10.6 ± 0.1^{b}	1.72 ± 0.12	1.53 ± 0.04^{y}	60.4 ± 5.3^{y}
	LP30-LY30	52.6 ± 1.0^{x}	$8.4 \pm 0.9^{c\,y}$	$10.2 \pm 0.4^{b\,x}$	1.89 ± 0.12	1.65 ± 0.06^{y}	58.6 ± 4.7^{y}

[1] Values (means ± SEM) with the same letter in the superscript within each colour (between diets; a, b, c) and within each diet (between colours; x, y) are not significantly different ($P > 0.05$; two-factor ANOVA, SNK). Where significant interactions occurred, values with the same letter in the superscript (in italics) within each colour (between diets; *a, b, c*) and within each diet (between colours; *x, y*) are not significantly different ($P > 0.05$; one-factor ANOVA, SNK).

[2] Diets were 0 mg carotenoids kg^{-1} (C0), 60 mg canthaxanthin kg^{-1} (LR60), 60 mg astaxanthin kg^{-1} (LP60), 60 mg apocarotenoic acid ethyl ester kg^{-1} (LY60), 30 mg canthaxanthin kg^{-1} + 30 mg apocarotenoic acid ethyl ester kg^{-1} (LR30-LY30), and 30 mg astaxanthin kg^{-1} + 30 mg apocarotenoic acid ethyl ester kg^{-1} (LP30-LY30).

[3] Data $\log_{10}$ transformed to homogenize variances.

TABLE 2
Results of ANOVA (*P* values) and *L**, *a**, *b** skin colour values and total skin carotenoids of snapper fed carotenoids and transferred to different cage colours after 7 or 14 days [1,2,3]. (Experiment 2)

	Cage colour	*Diet*	*Cage colour* x *Diet*
7 days			
*L**	$P < 0.001$	$P < 0.001$	ns
*a**	ns	$P < 0.001$	ns
*b**	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
14 days			
*L**	$P < 0.05$	ns	ns
*a**	ns	$P < 0.0001$	ns
*b**	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
Total carotenoids [4]	$P < 0.05$	$P < 0.0001$	$P < 0.01$

Cage colour	*Diet*	*L**	*a**	*b**	Total carotenoids (μg g^{-1}) [4]
7 days					
White	C0	$71.8 \pm 0.7^{c\,y}$	0.1 ± 0.9^{a}	7.2 ± 0.4^{a}	-
	LR60	$71.2 \pm 1.3^{bc\,y}$	2.0 ± 0.9^{a}	10.0 ± 0.5^{bc}	-
	LP60	$68.9 \pm 0.9^{ab\,y}$	5.1 ± 1.2^{b}	$12.7 \pm 0.5^{d\,y}$	-
	LY60	$70.4 \pm 0.4^{abc\,y}$	0.3 ± 0.7^{a}	6.9 ± 0.3^{a}	-
	LR30-LY30	$71.6 \pm 0.2^{bc\,y}$	2.0 ± 1.4^{a}	9.2 ± 0.7^{b}	-
	LP30-LY30	$68.1 \pm 0.6^{a\,y}$	4.6 ± 0.8^{b}	$11.2 \pm 0.3^{c\,y}$	-
Black-White	C0	$70.9 \pm 0.4^{c\,x}$	0.7 ± 1.1^{a}	7.9 ± 0.5	-
	LR60	$69.2 \pm 1.0^{bc\,x}$	1.7 ± 1.3^{a}	8.7 ± 0.6	-
	LP60	$65.8 \pm 2.0^{ab\,x}$	3.9 ± 0.8^{b}	8.9 ± 0.5^{x}	-
	LY60	$68.1 \pm 0.7^{abc\,x}$	0.9 ± 0.6^{a}	7.4 ± 0.4	-
	LR30-LY30	$67.8 \pm 1.2^{bc\,x}$	1.2 ± 1.0^{a}	8.2 ± 0.2	-
	LP30-LY30	$66.9 \pm 0.9^{a\,x}$	3.4 ± 0.8^{b}	9.2 ± 0.4^{x}	-
14 days					
White	C0	71.4 ± 1.0^{y}	1.0 ± 0.4^{a}	7.9 ± 0.3^{a}	3.66 ± 0.24^{a}
	LR60	67.9 ± 1.4^{y}	1.8 ± 0.4^{a}	$11.2 \pm 0.4^{c\,y}$	5.00 ± 0.44^{a}
	LP60	66.4 ± 2.6^{y}	6.5 ± 0.5^{b}	$13.3 \pm 0.4^{d\,y}$	$9.78 \pm 0.72^{b\,x}$
	LY60	71.4 ± 1.0^{y}	1.5 ± 0.6^{a}	7.9 ± 0.4^{a}	3.70 ± 0.19^{a}
	LR30-LY30	71.2 ± 1.1^{y}	1.2 ± 0.6^{a}	9.7 ± 0.6^{b}	4.77 ± 0.29^{a}
	LP30-LY30	68.5 ± 1.1^{y}	5.5 ± 0.5^{b}	$12.3 \pm 0.5^{cd\,y}$	9.54 ± 1.03^{b}
Black-White	C0	69.9 ± 0.9^{x}	0.9 ± 0.6^{a}	8.5 ± 0.2^{ab}	3.54 ± 0.20^{a}
	LR60	67.9 ± 1.4^{x}	1.6 ± 0.6^{a}	$9.1 \pm 0.3^{abc\,x}$	5.29 ± 0.28^{b}
	LP60	67.0 ± 0.7^{x}	5.7 ± 0.7^{b}	$10.1 \pm 0.2^{c\,x}$	$14.78 \pm 0.49^{d\,y}$
	LY60	68.2 ± 1.6^{x}	1.8 ± 0.4^{a}	7.8 ± 0.2^{a}	3.28 ± 0.12^{a}
	LR30-LY30	68.2 ± 1.8^{x}	1.8 ± 0.3^{a}	9.3 ± 0.6^{abc}	4.64 ± 0.47^{b}
	LP30-LY30	66.2 ± 1.5^{x}	5.2 ± 0.6^{b}	$10.0 \pm 0.6^{bc\,x}$	11.81 ± 1.02^{c}

[1] Values (means ± SEM) with the same letter in the superscript within each colour (between diets; a, b, c) and within each diet (between colours; x, y) are not significantly different ($P > 0.05$; two-factor ANOVA, SNK). Where significant interactions occurred, values with the same letter in the superscript (in italics) within each colour (between diets; *a, b, c, d*) and within each diet (between colours; *x, y*) are not significantly different ($P > 0.05$; one-factor ANOVA, SNK).
[2] Diets were 0 mg carotenoids kg^{-1} (C0), 60 mg canthaxanthin kg^{-1} (LR60), 60 mg astaxanthin kg^{-1} (LP60), 60 mg apocarotenoic acid ethyl ester kg^{-1} (LY60), 30 mg canthaxanthin kg^{-1} + 30 mg apocarotenoic acid ethyl ester kg^{-1} (LR30-LY30), and 30 mg astaxanthin kg^{-1} + 30 mg apocarotenoic acid ethyl ester kg^{-1} (LP30-LY30).
[3] Values of control snapper fed C0 diet and held in black cages at 7 days were $L^* = 58.8 \pm 0.8$, $a^* = 1.3 \pm 0.9$, $b^* = 6.6 \pm 0.4$, and at 14 days were $L^* = 55.4 \pm 2.7$, $a^* = 2.6 \pm 0.4$, $b^* = 7.2 \pm 0.4$, total carotenoids 3.62 ± 0.15 μg g^{-1}.
[4] Data $\log_{10}$ transformed to homogenize variances.

TABLE 3
Results of ANOVA (*P* values) and *L**, *a**, *b** skin colour values, feed conversion ratios (FCR), feed consumption (% biomass day^{-1}) and % weight gain of snapper held in different cage colours and fed different diets for 50 days [1,2]. (Experiment 3)

Variable	*Cage colour*	*Diet*	*Cage colour* x *Diet*
*L**	$P < 0.0001$	ns	ns
*a**	ns	$P < 0.0001$	ns
*b**	$P < 0.01$	$P < 0.0001$	$P < 0.01$
FCR [3]	ns	ns	ns
% biomass day^{-1}	$P < 0.05$	ns	ns
% weight gain	$P < 0.05$	ns	ns

Cage colour	*Diet*	*L**	*a**	*b**	FCR [3]	% biomass day^{-1}	% weight gain
White	C0	71.3 ± 1.9^{z}	2.9 ± 0.8^{a}	8.3 ± 0.4^{a}	1.64 ± 0.05	1.49 ± 0.05^{x}	60.29 ± 1.37^{x}
	LP60	68.4 ± 2.0^{z}	8.0 ± 0.4^{b}	13.7 ± 0.2^{by}	1.77 ± 0.13	1.45 ± 0.04^{x}	54.17 ± 4.35^{x}
Black	C0	55.3 ± 1.1^{x}	4.5 ± 0.6^{a}	8.4 ± 0.5^{a}	1.59 ± 0.08	1.59 ± 0.04^{y}	69.58 ± 4.98^{y}
	LP60	57.9 ± 2.6^{x}	8.9 ± 0.6^{b}	10.8 ± 0.4^{bx}	1.63 ± 0.11	1.63 ± 0.03^{y}	68.76 ± 4.22^{y}
Red	C0	63.2 ± 2.4^{y}	4.4 ± 0.6^{a}	8.6 ± 0.8^{a}	1.62 ± 0.05	1.54 ± 0.06^{xy}	63.72 ± 2.61^{xy}
	LP60	63.3 ± 1.5^{y}	8.4 ± 0.9^{b}	14.5 ± 1.0^{by}	1.58 ± 0.06	1.51 ± 0.04^{xy}	65.21 ± 3.30^{xy}
Blue	C0	64.0 ± 1.7^{y}	4.3 ± 0.6^{a}	8.6 ± 0.4	1.70 ± 0.09	1.63 ± 0.06^{y}	65.09 ± 2.27^{xy}
	LP60	63.0 ± 0.4^{y}	10.0 ± 0.6^{b}	10.4 ± 0.8^{x}	1.65 ± 0.11	1.56 ± 0.02^{y}	65.36 ± 5.14^{xy}

[1] Values (means ± SEM) with the same letter in the superscript within each colour (between diets; a,b) and within each diet (between colours; x, y, z) are not significantly different ($P > 0.05$; two-factor ANOVA, SNK). Where significant interactions occurred, values with the same letter in the superscript (in italics) within each colour (between diets; *a, b*) and within each diet (between colours; *x, y*) are not significantly different ($P > 0.05$; one-factor ANOVA, SNK).
[2] Diets were 0 mg carotenoids kg^{-1} (C0) and 60 mg astaxanthin kg^{-1} (LP60).
[3] Data $\log_{10}$ transformed to homogenize variances.

4.4 Effects of dietary astaxanthin concentration and feeding period on the skin pigmentation of Australian snapper *Pagrus auratus* (Bloch & Schneider, 1801)

Ben J. Doolan[1,2], Mark A. Booth[2], Geoff L. Allan[2], and Paul L. Jones[1]

[1] *School of Life and Environmental Sciences, Deakin University, Warrnambool, Victoria 3280, Australia*
[2] *NSW Department of Primary Industries, Port Stephens Fisheries Centre and Aquafin CRC, Private Bag 1, Nelson Bay NSW 2315*

ABSTRACT

A single factor experiment was conducted to investigate the effects of dietary astaxanthin concentration on the skin colour of snapper. Snapper (mean weight = 129 g) were held in white cages and fed one of 7 dietary levels of unesterified astaxanthin (0, 13, 26, 39, 52, 65 or 78 mg astaxanthin kg^{-1}) for 63 days. Treatments comprised four replicate cages, each containing five fish. The skin colour of all fish was quantified using the CIE *L**, *a**, *b** colour scale after 21, 42 and 63 days. In addition, total carotenoid concentrations of the skin of 2 fish $cage^{-1}$ were determined after 63 days. Supplementing diets with astaxanthin strongly affected redness (*a**) and yellowness (*b**) values of the skin at all sampling times. After 21 days, the *a** values increased linearly as the dietary astaxanthin concentration was increased before a plateau was attained between 39 and 78 mg kg^{-1}. The *b** values similarly increased above basal levels in all astaxanthin diets. By 42 days, *a** and *b** values increased in magnitude while a plateau remained between 39 and 78 mg kg^{-1}. After 63 days, there were no further increases in measured colour values suggesting that maximum pigmentation was imparted in the skin of snapper fed diets >39 mg kg^{-1} after 42 days. Similarly, there were no differences in total carotenoid concentrations of the skin of snapper fed diets >39 mg kg^{-1} after 63 days. The plateaus that occurred in *a** and *b** values, while still increasing in magnitude between 21 and 42 days, indicate that the rate of astaxanthin deposition in snapper is limited and astaxanthin in diets containing >39 mg astaxanthin kg^{-1} is not efficiently utilized. Astaxanthin retention after 63 days was greatest from the 13 mg kg^{-1} diet; however, skin pigmentation was not adequate. An astaxanthin concentration of 39 mg kg^{-1} provided the second greatest retention in the skin while obtaining maximum pigmentation. To efficiently maximize skin pigmentation, snapper growers should commence feeding diets containing a minimum of 39 mg unesterified astaxanthin kg^{-1} at least 42 days before sale.

1. INTRODUCTION

Carotenoids are used in aquafeeds for finfish to enhance the colour of the flesh or skin. However, carotenoid supplements are expensive and their addition to feeds can account for 10–15% (Buttle, Crampton & Williams, 2001) to 20–5% (Torrissen, Christiansen, Struksnæs & Estermann, 1995) of total feed costs for farmers. Astaxanthin is the most common carotenoid pigment used in finfish feeds and has been identified as the main carotenoid responsible for pink skin pigmentation in snapper (Doolan, Allan, Booth & Jones, in press [Chapter 4.3]), red sea bream *Pagrus major* (Katayama, Ikeda & Harada, 1965; Tanaka, Katayama, Simpson & Chichester, 1976) and red porgy *Pagrus pagrus* (Kalinowski, Robaina, Fernández-Palacios, Schuchardt & Izquierdo, 2005; Chatzifotis, Pavlidis, Jimeno, Vardanis, Sterioti & Divanach, 2005). In Australia, snapper fed diets without astaxanthin supplementation exhibit a dull grey appearance that leads to reduced consumer acceptance and subsequently lower wholesale and retail prices (Doolan, Booth, Jones & Allan, 2007 [Chapter 4.2]). Adding astaxanthin to feeds and improving its retention in the skin of snapper may provide economic benefits to snapper farmers by optimizing the value of their fish.

Several models of astaxanthin utilization have been proposed in salmonids (Torrissen, Hardy & Shearer, 1989). The first assumes that retention of astaxanthin in the flesh increases linearly with increasing fish weight, implying that high concentrations in the diet should be supplied before harvest. The second model suggests constant retention efficiency regardless of fish size; therefore, diets low in

astaxanthin should be fed throughout production. Preliminary research with snapper indicates that astaxanthin can be deposited in the skin quickly (1–2 months; Booth, Warner-Smith, Allan & Glencross, 2004 [Chapter 4.1]; Doolan, *et al.* in press [Chapter 4.3]) so the first mode of retention may apply.

Generally, providing a biologically available source of carotenoid is supplied, carotenoid content in salmonid tissue increases linearly until it reaches a plateau level (Storebakken, Foss, Huse, Wandsvik & Lea, 1986; Torrissen, 1986; Storebakken, Foss, Schiedt, Austreng, Liaaen-Jensen & Manz, 1987; Choubert & Storebakken, 1989; Torrissen, *et al.* 1995). Therefore, higher dietary concentrations lead to poorer retention in the flesh once the plateau has been reached. Torrissen, *et al.* (1995) hypothesized an equilibrium between plasma and flesh astaxanthin concentrations of Atlantic salmon *Salmo salar*, therefore, a high flesh concentration could limit absorption of astaxanthin into the bloodstream. In contrast, earlier work by Torrissen (1985) reported greater flesh deposition of astaxanthin with increasing dietary astaxanthin concentration in rainbow trout *Oncorhynchus mykiss* over 240 days with no such plateau, although retention efficiency decreased as dietary concentration increased. Of the few snapper studies conducted with astaxanthin, Booth, *et al.* (2004 [Chapter 4.1]) and Doolan, *et al.* (in press [Chapter 4.3]) suggested that a similar process may occur in this species with limitations in astaxanthin deposition in the skin when fed diets containing astaxanthin >36 mg kg^{-1} after 42 days, and 30 mg kg^{-1} after 51 days, respectively; however, few astaxanthin levels were tested.

Many factors can affect the rate and efficiency of carotenoid deposition in finfish including dietary carotenoid level, duration of feeding, dietary lipid content, fish size, stage of maturity and even season (Torrissen, *et al.* 1995). This study aimed to identify the optimal dietary level of unesterified astaxanthin to produce maximum skin pigmentation in immature snapper after 3, 6 and 9 weeks of feeding.

2. MATERIALS AND METHODS

2.1 Experimental design and colour measurement

A single-factor experiment was conducted at the Port Stephens Fisheries Centre (PSFC) to determine the effect of astaxanthin inclusion level (0, 13, 26, 39, 52, 65 or 78 mg astaxanthin kg^{-1}; diets denoted as C0, LP13, LP26, LP39, LP52, LP65 and LP78, respectively) in the diet on snapper skin colour after 21, 42 and 63 days of feeding. Each treatment was replicated with four cages stocked with five snapper. At 63 days, two fish from each cage were slaughtered to quantify total carotenoid concentrations in skin and flesh samples.

Colour was quantified using the CIE *L**, *a**, *b** colour scale advised by the International Commission on Illumination where *L** represents lightness (0 = black to 100 = white), *a** represents green (-60) to red (+60) and *b** represents blue (-60) to yellow (+60). Skin colour was measured using a handheld chroma-meter (CR-10, Minolta, UK; Selby Biolab, Gladesville, NSW, Australia) on three separate positions along the left flank of fish. Values from the three positions were averaged as described by Booth *et al.* (2004 [Chapter 4.1]) to provide single *L**, *a** and *b** values for each fish.

2.2 Diets

Seven experimental diets were prepared after re-milling the MF60 diet (46% crude protein, 19% fat; Ridley Agriproducts, Narangba, Qld, Australia) used by Booth, Allan and Anderson (submitted). The diet was ground through a hammer mill (Raymond Laboratory Mill, Transfield Technologies, Rydalmere, Australia) fitted with a 1.5 mm screen and mixed with 50 g kg^{-1} 100% pre-gelatinised wheat starch (Pregel N, Penford Australia, Lane Cove, NSW, Australia) to assist in re-binding. The mash was thoroughly re-mixed with the desired inclusion level of unesterified astaxanthin (LP; Lucantin® Pink, 10% formulation, BASF, Germany) to develop the test diets and distilled water was added to form pellets through a meat mincer (Barnco Australia, Leichardt, NSW, Australia) fitted with a 4 mm die. The same procedure was applied to the basal diet (C0). Diets were dried at 35– 0°C for 3–

4 h until moisture contents was <10% to produce a dry, slow-sinking pellet and stored at <-15^{o}C until required.

2.3 Facilities

A saltwater recirculating system located within a hothouse facility consisting of pale blue fibreglass 10,000 L tanks (3.4 m diameter; 1.1 m deep) was used to house the experiment cages. Eight floating white (L^* = 87.6, a^* = 0.9, b^* = 5.1) circular 0.2 m^3 cages (600 mm diameter; 700 mm depth) were placed around the perimeter of each tank and supplied with recirculated, filtered (15 μm) water through individual 15 mm polyethylene pipes at a flow rate of approximately 2.5 L min^{-1} cage^{-1}. Cage walls were constructed of 420 μm PVC plastic consisting of a woven polyester base fabric (Ricky Richards, Homebush, NSW) and bases with a 4 mm white oyster mesh to enable passage of faecal wastes and additional water exchange. Tanks were vacuum siphoned every second day and cartridge filters were exchanged daily to remove accumulated wastes from the system. Tanks were aerated with 4 x 100 mm air stone diffusers and temperatures were maintained by 1 kW immersion heaters.

Water quality was monitored daily using a water quality analyser (Model 611, Yeo-Kal Electronics, Brookvale, NSW, Australia). Total ammonia was monitored colourimetrically twice weekly (Model 1.08024, E. Merck, Darmstadt, Germany). Temperature, pH, salinity, and dissolved oxygen where in the range 17.01 – 26.63^{o}C, 7.68 – 8.41, 29.98 – 34.27 g L^{-1} and 6.0 – 8.5 mg L^{-1} respectively, and total ammonia was always <0.4 mg L^{-1}.

2.4 Fish and feeding

The juvenile snapper (128.6 ± 8.6 g; mean ± SD, n = 140) used in this study were approximately 12 months old and were progeny of first-generation broodstock produced at PSFC. All fish were grown in pale blue (L^* = 73.3, a^* = -11.5, b^* = 3.1) 10 000 L tanks and fed twice daily on a commercial barramundi *Lates calcarifer* diet (50% protein, 12% fat; Barra Grower 18MJ, Ridley Agriproducts, Narangba, Qld, Australia) before the experiment.

Upon stocking the experiment, snapper were anaesthetized with 25 mg L^{-1} ethyl-*p*-aminobenzoate. Fish were randomly removed, individually weighed and measured for colour before distribution into experimental cages (5 fish cage^{-1}). Six randomly selected fish were slaughtered for determination of initial carotenoid content. Snapper were fed with test diets to apparent satiation twice daily at 09:00 and 15:00 hours (Australian Eastern Standard Time) and starved for 1 day before sampling. Fish were anaesthetized *in situ* before colour and weight assessments at days 21, 42 and 63 using the protocol described above. After 63 days, two fish from each cage were killed for carotenoid analysis with a lethal dose of ethyl-*p*-aminobenzoate (100 mg L^{-1}). The left flank of fish was removed, vacuum packaged (-0.8 bar; Genius^{TM}, Vacuseal, Caboolture, Qld, Australia) and stored in the dark at <-15^{o}C until required.

2.5 Carotenoid extraction and analysis

Frozen samples were slowly thawed and the skin and flesh separated with a knife. Carotenoid extractions from skin and flesh samples were carried out using the method of Cejas, Almansa, Tejera, Jerez, Bolannos and Lorenzo (2003) modified from Barua, Kostic and Olson (1993). Samples were finely cut with scissors, weighed and skin (0.5 – 0.9 g) or flesh (2.1 – 3.1 g) was homogenized in 10 mL ethyl acetate:ethanol (1:1 v/v) and left to extract for 15 min. The mixture was vortexed and centrifuged [6,000 rpm (4000 *g*) 2 min] and the supernatant was removed. The pellet was extracted twice more, with 5 mL ethyl acetate followed by 10 mL hexane. The three supernatants were pooled and evaporated to dryness under vacuum. Samples were resuspended in 2 mL hexane containing 0.02% BHT (w/v) (Torrissen, 1995) and centrifuged [15,000 rpm (5000 *g*) 4 min]. The total carotenoid concentration was quantified spectrophotometrically in hexane (containing 0.02% BHT) at 470 nm using $E_{1\%1cm}$ = 2100.

2.6 Astaxanthin retention calculations

Carotenoid retention coefficients or retention ratios are typically used to demonstrate the efficiency of carotenoid deposition in the flesh to carotenoids provided in the feed in dose-response studies in salmonids (Torrissen, 1985, Choubert & Storebakken, 1989; Smith, Hardy & Torrissen, 1992; Torrissen, 1995; Torrissen, *et al.* 1995; Buttle, *et al.* 2001). However, there is no available literature on quantification of carotenoid retention in sparid studies where carotenoids are deposited in the skin. Torrissen, *et al.* (1995) showed that retention coefficients can be calculated in the skin of Atlantic salmon *Salmo salar* when evaluating whole fish retention coefficients (muscle, gonads, plasma and skin). Because of the difficulty involved in removing and obtaining the weight of the entire skin of the fish, and because total carotenoids were quantified from the flank of the fish only, retention coefficients were not used in this study. Rather, the following basic relative retention ratio was devised for comparative purposes between treatments:

$$R = (1000)\ (Ax_f - Ax_i) / AxF$$

where Ax_f is the final astaxanthin concentration (μg g^{-1}), Ax_i is the initial astaxanthin concentration (μg g^{-1}) and $A\ x\ F$ is average total astaxanthin consumption $fish^{-1}$ (μg) throughout the feeding period. Higher ratios indicate more efficient deposition of astaxanthin in the skin of snapper.

2.7 Statistical analyses and calculations

Data were tested for homogeneity of variance using Cochran's test before one-factor ANOVA (where cages were treated as replicates) using STATGRAPHICS PLUS Version 4.1 (Manugistics, Rockville, MD, USA). When heterogeneous, data were $\log_{10}$ or $\log_{10} + 1$ transformed to homogenize variances. Because of repeat sampling of fish, data from each sampling period were analysed separately. The Student-Newman-Keuls (SNK) test was used to separate significant treatment means when required ($P<0.05$). Non-linear regression analyses were used to investigate relationships between colour values and total carotenoid concentrations at 63 days.

3. RESULTS

Skin lightness (L^*) was not significantly affected ($P>0.05$) by diet after 21, 42, or 63 days (Figure 1). However, there was a general darkening (decrease in L^*) with increasing a^* and b^* values after feeding astaxanthin.

The inclusion of astaxanthin in feeds had a strong effect on skin redness (a^*) and yellowness (b^*) at all sampling times following the commencement of feeding (Figure 1). At day 21, a^* values increased as the dose rate was increased until a plateau was attained from LP39 to LP78. Maximum skin redness (a^*) was achieved after 42 days, with an increase in a^* values observed in fish from all astaxanthin-supplemented diets, with a plateau remaining apparent beyond LP39. By day 63, all a^* values of snapper fed LP39 to LP78 had decreased, and the plateau appeared from the lower concentration of LP26 upwards to LP78. In general, b^* values followed a similar trend to a^* values (Figure 1). At day 21, all fish fed the LP diets displayed higher b^* values than fish fed the basal diet (C0); however, there were no differences in b^* values between LP13 and LP78 diets. A dose-dependent response occurred in b^* by day 42, with a plateau beyond LP39. The decrease observed in a^* between days 42 and 63 did not occur in b^*. b^* values of fish fed diets from LP39 to LP78 remained similar at day 63 to day 42; however, a large increase occurred in the lower astaxanthin treatments of LP13 and LP26.

The total carotenoid concentration of the skin of snapper increased above basal levels in all fish supplemented with astaxanthin after 9 weeks of feeding (Table 1). A distinct plateau was apparent between diets containing LP39 to LP78 at a concentration of approximately 12 μg g^{-1} in the skin.

Estimations of total carotenoids from colour variables after 63 days were best approximated by a^* = 2.37 Ln (carotenoid concentration) - 0.72, R^2 = 0.61; and b^* = 2.13 Ln (carotenoid concentration) + 10.00, R^2 = 0.54.

The retention of carotenoids in the skin of snapper was greatest in fish fed LP13, and LP39 (Table 1). While fish supplied with greater astaxanthin concentrations in the diet ingested the most astaxanthin over the 63 days of the experiment (Table 1), retention in the skin was lowest from these diets.

There were no differences (P>0.05) in the total carotenoid concentrations of the flesh of snapper between all diets fed after 63 days, with all flesh samples quantified containing <0.27 μg g^{-1} total carotenoids.

There were no differences in survival (98%), daily feed consumption, weight gain or feed conversion ratios between diets during any time period or over the entire feeding trial (Table 1).

4. DISCUSSION

Unesterified astaxanthin was an effective dietary supplement for enhancing pigmentation in Australian snapper based on L^*, a^*, b^* colour values and skin carotenoid concentration, similar to the findings of Doolan, *et al.* (in press [Chapter 4.3]). Traditionally, esterified astaxanthin has been readily accepted as being more efficiently utilized than unesterified astaxanthin in the closely related *P. major* (Nakazoe, Ishii, Kaminoto & Takeuchi, 1984; Ito, Kamata, Tanaka & Sameshima, 1986; Ha, Kang, Kim, Choi & Ryu, 1993). However, unesterified astaxanthin in the diet of snapper provided adequate pigmentation beyond 42 days and has previously been shown to be as effective as esterified astaxanthin in snapper (Booth, *et al.* 2004 [Chapter 4.1]). The similarities in a^* and b^* values of the skin of snapper fed the 39 to 78 mg astaxanthin kg^{-1} (LP39 – LP78) diets at days 21, 42 and 63 indicated that 39 mg unesterified astaxanthin kg^{-1} was as effective as twice that amount for maximum skin pigmentation at all sampling times. This demonstrates that feeding diets that contain more than 39 mg astaxanthin kg^{-1} is not necessary for enhancing skin pigmentation in snapper.

From our findings, it appears that the rate of astaxanthin deposition and/or absorption in snapper may be limited. The plateaus observed at each sampling time may indicate that a threshold in the ability to deposit astaxanthin was reached or that transportation to the skin was saturated as suggested by Storebakken, *et al.* (1987) with *S. salar*. The plateau in skin a^* and b^* values obtained during the first sampling period (0–21 days) increased in magnitude over the subsequent 21 days as the fish likely deposited more astaxanthin in the skin. However, fish fed greater than 39 mg astaxanthin kg^{-1} likewise did not show an increase the rate of deposition and the observed skin a^* and b^* values were similar. Based on a^* and b^* values, the increase by 63 days in snapper fed 26 mg astaxanthin kg^{-1} (LP26) to the level of fish fed 39–78 mg astaxanthin kg^{-1}, with no further increase from those diets, indicates that a saturation level of astaxanthin deposited in the skin of fish fed 39, 52, 65 and 78 mg astaxanthin kg^{-1} had been attained by 42 days, 26 mg astaxanthin kg^{-1} in the feed took 63 days, while feeding 13 mg astaxanthin kg^{-1} did not reach astaxanthin saturation in the skin by 63 days with the feeding regime applied in this study. However, the total carotenoid concentrations of snapper fed LP26 remained significantly lower after 63 days.

Similar findings in colour values have been previously reported in snapper fed unesterified astaxanthin by Booth, *et al.* (2004 [Chapter 4.1]) with 36 mg astaxanthin kg^{-1} (supplied as Carophyll™ Pink; Hoffmann-La Roche, Basel, Switzerland) as effective as 72 mg kg^{-1} after 42 days, and Doolan *et al.* (in press [Chapter 4.3]) with 30 mg astaxanthin kg^{-1} (supplied as Lucantin® Pink, BASF) as effective as 60 mg kg^{-1} after 51 days. While Booth, *et al.* (2004 [Chapter 4.1]) only observed astaxanthin-dependent effects on a^* values, b^* values of their fish were much lower than our study likely Because of the darker skin colouration they observed, as measurable b^* values are higher in snapper fed astaxanthin in white cages (Doolan *et al.* in press [Chapter 4.3]).

Although not significantly different, lightness (L^*) values of snapper fed the basal diet were higher than those of snapper from all unesterified astaxanthin treatments. This was similar to the results of Doolan, *et al.* (in press [Chapter 4.3]) and comparable to the findings of Smith, *et al.* (1992) in the flesh of coho salmon *Oncorhynchus kisutch* and Christiansen, Struksnæs, Estermann and Torrissen (1995) in *S. salar*. These visual differences in the skin of snapper were likely caused from the erythrophores that contain astaxanthin esters in the skin (Lin, Ushio, Ohshima, Yamanaka & Koizumi, 1998). While erythrophores obviously affect a^* and b^* values, they also slightly darken the skin, but not to the extent of melanophores. While high L^* values are preferential in snapper, lower L^* values should be expected when supplementing astaxanthin in diets (Doolan, *et al.* in press [Chapter 4.3]).

Higher a^* values were observed in the Lucantin® Pink treatments (LP39 – LP78) on day 42 than at day 63 when it was expected that the fish would not lose colouration with the additional 21 days of food consumption. Similar findings have been reported in *P. pagrus* (Kalinowski, *et al.* 2005). Although the same sampling protocol was used at all colour measurements and water quality parameters were almost identical, snapper likely altered the pink appearance by aggregating erythrosomes within erythrophore cells to appear less vivid (Fujii, 1993). This may have been a reaction to changes in natural light intensity. Doolan, *et al.* (2007 [Chapter 4.2]) observed that the skin lightness (i.e. from melanophores) of snapper devoid of dietary astaxanthin was not significantly affected by light intensity when situated in either black or white cages; however, erythrophores may be more sensitive to light intensity in snapper under the experimental conditions. Because of such temporal skin colour changes in snapper, estimating astaxanthin concentration from a^* values in particular is difficult, as experienced with snapper exposed to different background colours (Doolan, *et al.* in press [Chapter 4.3]), and it is recommended that snapper be analysed for carotenoid determination after each sampling period as performed by Storebakken, *et al.* (1987), Choubert and Storebakken (1989), Torrissen, *et al.* (1995), Gomes, Dias, Silva, Valenta, Empis, Gouveia, Bowen and Young (2002) and Gouveia, Choubert, Pereira, Santinha, Empis and Gomes (2002) in trials that focus on carotenoid dose responses with time.

These changes in a^* values suggest that carotenoid concentration of the integument does not necessarily equate to vivid skin pigmentation. As such, the observable skin colour of snapper (as indicated by L^*, a^* and b^* values) may be more important in studies for improving the marketability of snapper than the carotenoid concentration of the skin, in contrast with the closely correlated relationships between observable colour and astaxanthin concentration in the flesh of salmonids (Storebakken, *et al.* 1987; Smith, *et al.* 1992; Christiansen, *et al.* 1995) where both methods can be accurately applied.

Growth rates were not affected by astaxanthin in the diet as previously reported in *P. pagrus* (Kalinowski, *et al.* 2005) and snapper (Doolan, *et al.* in press [Chapter 4.3]). The moderately high feed conversion ratios during the experiment were a result of the loss of dry matter during feeding, caused primarily by antagonistic behaviour between individuals during the earlier stages of the experiment. Dominant fish, observed in most cages, consumed excessive quantities of pellets before expelling them, or harassed other fish causing them to expel feed. Stocking densities higher than the ~3 kg m^{-3} in the current trial may alter these social interactions and reduce such antagonistic behaviour (Sayer, 1998). As this can lead to improved growth rates, salmonids can deposit carotenoids more quickly (Hatlen, Arnesen, Jobling, Siikavuopio & Bjerkeng, 1997; Jobling, Tveiten & Hatlen, 1998) or in snapper, may support the findings of a predefined astaxanthin deposition rate in the skin as observed in this study. In our study, snapper fed more vigorously during the afternoon feeds. Later feeding and higher feeding frequencies can increase growth rates and feed conversions of juvenile snapper (Tucker, Booth, Allan, Booth & Fielder, 2006). Varying feeding regimes warrant further investigation to verify the presumptions in the mode of astaxanthin deposition in snapper from this study.

The total carotenoid content in the flesh of snapper was negligible in accordance with the findings of Booth, *et al.* (2004 [Chapter 4.1]) remaining <0.27 μg g^{-1} and similar between all diets after 63 days. Such low levels indicate that astaxanthin is preferentially absorbed in the skin of immature snapper

with excess astaxanthin (as experienced when fed >39 mg astaxanthin kg^{-1}) likely lost through metabolic loss or excreted rather than deposited in the flesh.

Based on the total carotenoid concentration after 63 days of feeding, the efficiency of astaxanthin retention in the skin of snapper decreased as the level of astaxanthin in the diet was increased in accordance with salmonid studies (Torrissen, 1985; Choubert & Storebakken, 1989; Torrissen, *et al.* 1995). The highest ratio of gained astaxanthin concentration in the skin to astaxanthin consumed was obtained from 13 mg kg^{-1} (LP13); however, this concentration did not cause saturation in carotenoid deposition nor in *a** and *b** values. The second most efficient diet was 39 mg kg^{-1} (LP39), which led to the maximum skin carotenoid concentration and *a** and *b** values. As such, 39 mg kg^{-1} astaxanthin is recommended to efficiently produce maximal skin pigmentation in snapper.

In conclusion, we recommend supplementing snapper diets with approximately 39 mg unesterified astaxanthin kg^{-1} (as Lucantin® Pink) for 42 days. While no further improvement in skin colour was attained by 63 days, the 39 mg kg^{-1} diet provided the most efficient retention in the skin while attaining maximal observable skin pigmentation.

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TABLE 1
Interim and total average daily feed consumption, weight gain, feed conversion ratios and astaxanthin consumption, and final relative retention ratios and total carotenoid concentrations of snapper fed increasing levels of astaxanthin for 63 days.[1]

Diet[2]	Feed consumption (% biomass days^{-1})[3]	Wt gain fish^{-1} (g)	FCR[4]	Astaxanthin fish^{-1} (mg)[5]	Relative retention ratio[6]	Total carotenoids (ug g^{-1})[7]
0 - 21 days[8]						
C0	1.06 ± 0.03	11.20 ± 2.18	3.01 ± 0.47	0 ± 0 [a]	—	—
LP13	1.16 ± 0.05	12.03 ± 1.32	2.89 ± 0.42	0.43 ± 0.02 [b]	—	—
LP26	1.09 ± 0.11	13.96 ± 1.19	2.32 ± 0.19	0.83 ± 0.06 [c]	—	—
LP39	1.15 ± 0.05	15.33 ± 1.46	2.20 ± 0.14	1.29 ± 0.06 [d]	—	—
LP52	1.18 ± 0.05	17.12 ± 0.98	2.00 ± 0.14	1.77 ± 1.00 [e]	—	—
LP65	1.03 ± 0.04	10.33 ± 2.30	3.18 ± 0.54	1.90 ± 0.06 [e]	—	—
LP78	1.16 ± 0.08	11.80 ± 4.06	3.46 ± 0.70	2.59 ± 0.20 [f]	—	—
22 - 42 days[9]						
C0	1.60 ± 0.03	20.61 ± 1.37	2.45 ± 0.14	0 ± 0 [a]	—	—
LP13	1.60 ± 0.10	23.71 ± 2.86	2.28 ± 0.28	0.67 ± 0.04 [b]	—	—
LP26	1.51 ± 0.06	24.10 ± 1.90	2.05 ± 0.10	1.27 ± 0.04 [c]	—	—
LP39	1.48 ± 0.08	24.10 ± 1.24	2.02 ± 0.04	1.90 ± 0.11 [d]	—	—
LP52	1.54 ± 0.02	26.98 ± 0.85	1.90 ± 0.04	2.67 ± 0.03 [e]	—	—
LP65	1.56 ± 0.09	26.99 ± 1.54	`	3.28 ± 0.19 [f]	—	—
LP78	1.59 ± 0.08	22.83 ± 1.47	2.27 ± 0.19	3.99 ± 0.20 [g]	—	—
43 - 63 days						
C0	1.15 ± 0.03	22.93 ± 2.11	1.84 ± 0.12	0 ± 0 [a]	—	—
LP13	1.14 ± 0.09	23.13 ± 2.29	1.86 ± 0.09	0.56 ± 0.05 [b]	—	—
LP26	1.13 ± 0.05	22.74 ± 0.97	1.86 ± 0.10	1.10 ± 0.05 [c]	—	—
LP39	1.06 ± 0.05	21.49 ± 0.98	1.85 ± 0.04	1.55 ± 0.07 [d]	—	—
LP52	1.13 ± 0.05	26.76 ± 1.81	1.66 ± 0.03	2.31 ± 0.13 [e]	—	—
LP65	1.12 ± 0.08	27.22 ± 2.73	1.58 ± 0.04	2.78 ± 0.02 [f]	—	—
LP78	1.10 ± 0.06	21.46 ± 2.29	1.93 ± 0.11	3.18 ± 0.19 [g]	—	—
Total 0 - 63 days						
C0	1.25 ± 0.04	54.74 ± 4.63	2.27 ± 0.15	0 ± 0 [a]	—	1.30 ± 0.13 [a]
LP13	1.27 ± 0.07	58.88 ± 6.11	2.22 ± 0.21	1.66 ± 0.10 [b]	2.06 ± 0.23 [c]	5.16 ± 0.41 [b]
LP26	1.23 ± 0.04	60.80 ± 2.38	2.03 ± 0.07	3.20 ± 0.08 [c]	1.31 ± 0.13 [ab]	5.91 ± 0.38 [b]
LP39	1.21 ± 0.04	60.92 ± 2.55	2.00 ± 0.01	4.74 ± 0.18 [d]	1.94 ± 0.25 [bc]	10.97 ± 1.25 [c]
LP52	1.26 ± 0.02	70.86 ± 3.47	1.84 ± 0.06	6.74 ± 0.21 [e]	1.51 ± 0.14 [abc]	11.89 ± 0.69 [c]
LP65	1.20 ± 0.05	64.53 ± 4.14	1.90 ± 0.05	7.96 ± 0.43 [f]	1.20 ± 0.11 [a]	11.32 ± 1.09 [c]
LP78	1.25 ± 0.05	56.09 ± 5.36	2.28 ± 0.21	9.76 ± 0.45 [g]	1.09 ± 0.17 [a]	12.24 ± 1.49 [c]

[1] Values (means ± SEM, n = 4 cages) with no letters or displaying the same letter in the superscript within each time frame are not significantly different ($P > 0.05$; one-factor ANOVA, SNK).
[2] Letters denote control (C) or Lucantin® Pink (LP) diet. Numbers denote dietary astaxanthin concentration (mg kg^{-1}).
[3] Average daily feed consumption (% biomass day^{-1}) = [100 x (total dry feed intake / time) / (final – initial biomass / 2)].
[4] Feed conversion ratio (FCR) = [dry weight feed / wet weight gain fish]
[5] Astaxanthin consumption fish^{-1} based on feed intake and initial astaxanthin concentrations incorporated in feeds
[6] Relative retention ratio (R) = [(1000 x final skin astaxanthin concentration – initial skin astaxanthin concentration) / astaxanthin consumed]
[7] Initial total carotenoids (mean ± SD, n = 6 fish) = 1.74 ± 0.47 ug g^{-1}.
[8] Weight gain data Log_{10} transformed and astaxanthin data Log_{10} (astaxanthin + 1) transformed to homogenize variances.
[9] FCR data Log_{10} transformed to homogenize variances.

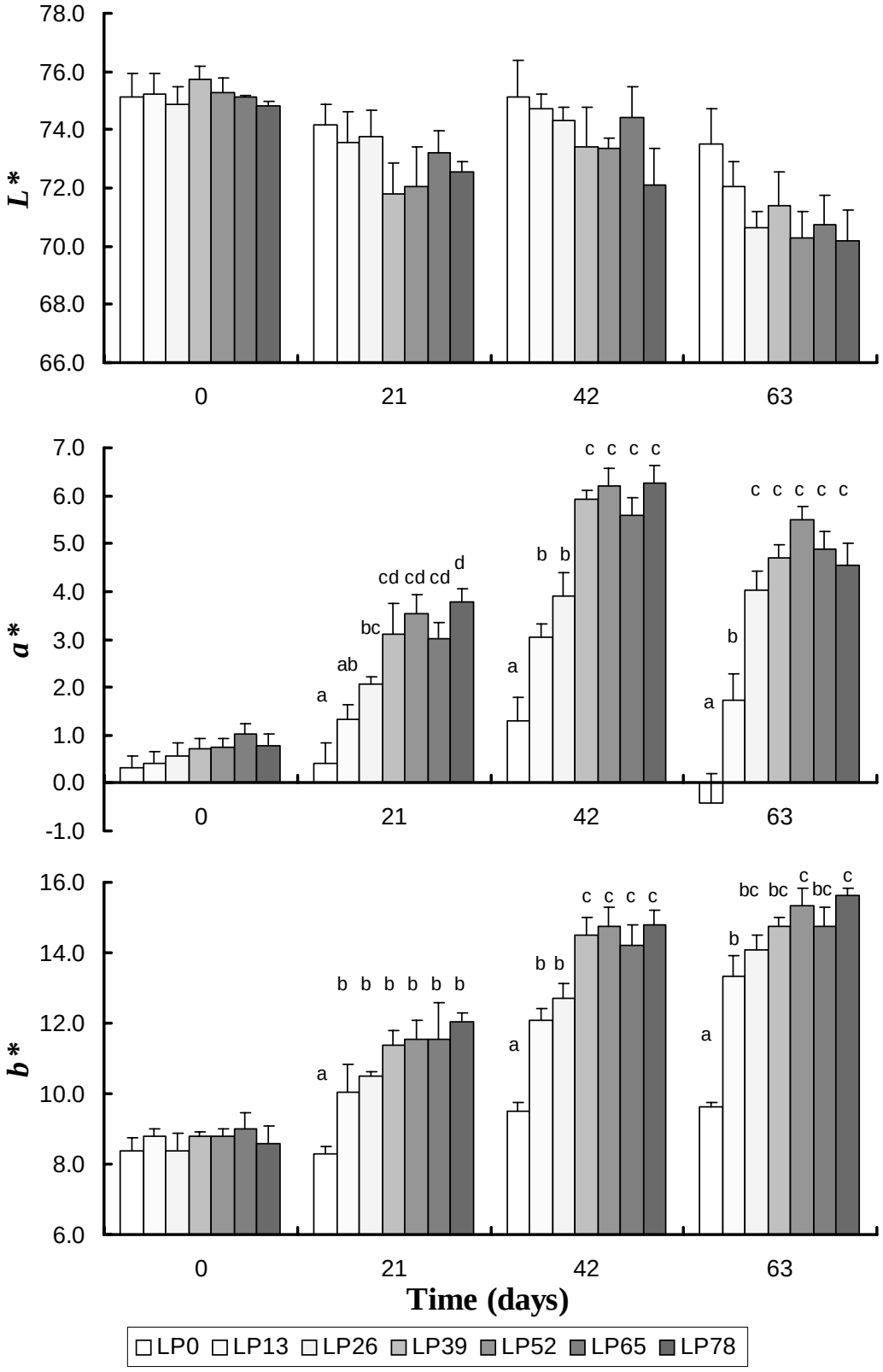

FIGURE 1
*L**, *a**, *b** values (means ± SEM, n = 4 cages) of snapper supplemented with Lucantin® Pink (LP) diets. Means with no letters or containing the same letter within each time are not significantly different (P>0.05; one-factor ANOVA, SNK).

4.5 Changes in the skin colour and cortisol response of Australian snapper *Pagrus auratus* to different background colours

Ben J. Doolan[1,2], Mark A. Booth[2], Geoff L. Allan[2], and Paul L. Jones[1]

[1] *School of Life and Environmental Sciences, Deakin University, Warrnambool, Victoria 3280, Australia*
[2] *NSW Department of Primary Industries, Port Stephens Fisheries Centre and Aquafin CRC, Private Bag 1, Nelson Bay NSW 2315, Australia*

ABSTRACT

A two-factor experiment was carried out to investigate the change in skin colour and plasma cortisol response of cultured Australian snapper *Pagrus auratus* to a change in background colour. Snapper (mean weight = 437 g) were held in black or white tanks and fed diets containing 39 mg unesterified astaxanthin kg^{-1} for 49 days before transferring from white tanks to black cages (WB) or black tanks to white cages (BW). *L** (lightness), *a** (redness) and *b** (yellowness) skin colour values of all snapper were measured at stocking (t = 0 days) and from cages of fish randomly assigned to each sampling time at 0.25, 0.5, 1, 2, 3, 5 and 7 days. Plasma cortisol was measured in anaesthetised snapper following colour measurements at 0, 1 and 7 days. Fish from additional black to black (BB) and white to white (WW) control treatments were also sampled for colour and cortisol at those times. Rapid changes occurred in skin lightness (*L** values) after altering background colour with maximum change in *L** values for BW and WB treatments occurring within one day. Skin redness (*a**) of BW snapper continued to steadily decrease over the 7 days ($a^* = 7.93\ e^{-0.051\ time}$). Plasma cortisol concentrations were highest at stocking when fish were held at greater densities and were not affected by cage colour. The results of this study suggest that transferring dark coloured snapper to white cages for one day is sufficient to affect the greatest benefit in terms of producing light coloured fish while minimising the reduction in favourable red skin colouration.

1. INTRODUCTION

Minimising the dark appearance of Australian snapper is a way of reducing consumer resistance in Australian markets to an otherwise excellent product. Implementing practical methods such as heavy shading to overcome the dark pigmentation of snapper when held in sea cages (Matsui, Tanabe, Furuichi, Yoshimatsu & Kitajima, 1992; Wakazono, Okajima, Shirai, Kitajima, Matsui, Tanabe & Matsumoto,1992; Booth, Warner-Smith, Allan & Glencross, 2004 [Chapter 4.1]) is difficult and inefficient compared with holding snapper in white tanks or white solid-walled cages (Doolan, Booth, Jones & Allan, 2007 [Chapter 4.2]; Doolan, Allan, Booth & Jones, in press a [Chapter 4.3]). Furthermore, maintaining white cages during sea cage farming operations is also difficult partly Because of problems with fouling changing the colour of nets (Doolan, Allan, Booth & Jones, in press b [Chapter 4.7]). One approach is to transfer snapper to white or pale coloured tanks before sale to increase skin lightness and improve market value. Obviously, a rapid turnover of fish through such a process would be essential and therefore determining the minimum time needed is important. Rapid changes (2 days) in skin lightness have been observed in snapper but these fish were fed diets devoid of astaxanthin and lacked the red colouration that is also desired by purchasers in snapper (Doolan, *et al.* 2007 [Chapter 4.2]).

It is also important to understand how rapidly pale coloured snapper will darken once transferred to black cages, a potential situation in a sea cage snapper growing operation. Teleosts commonly alter their appearance to blend in with the surrounding environment using cryptic colouration (see review by Fujii, 1993a). Studies investigating skin colour changes in sparids typically report colour data on one occasion (Szisch, Van der Salm, Wendelaar Bonga & Pavlidis, 2002; Doolan, *et al.* in press b [Chapter 4.7]) or at irregular intervals (Van der Salm, Martínez, Flik & Wendelaar Bonga, 2004; Doolan *et al.* 2007 [Chapter 4.2]; Doolan *et al.* in press a [Chapter 4.3]) despite physiological changes

in the motility of melanosomes within melanophores in response to changes in background colour observed within 30 seconds (Pavlidis, Karkana, Fanouraki & Papandroulakis, in press). There is currently no available published information on the ongoing daily changes in skin colour to changes in background colour after the initial rapid physiological change. This information is of interest because Sugimoto (1993) observed additional morphological changes in the melanophores of medaka *Oryzias latipes* after exposure to black or white backgrounds for 10 days and similar changes may emerge in snapper under these conditions.

While transferring dark coloured snapper to white cages has previously been shown to dramatically increase skin lightness (Doolan, *et al.* in press a [Chapter 4.3]), such environmental alterations may lead to the activation of stress responses in the fish. Cortisol is the most commonly used indicator of stress in fish (Wendelaar Bonga, 1997) and has been studied extensively in snapper (Pankhurst & Sharples, 1992; Lowe, Ryder, Carragher & Wells, 1993; Cleary, Pankhurst & Battaglene, 2000). Importantly, Lowe *et al.* (1993) observed poorer flesh quality in stressed snapper that had elevated cortisol levels pre-mortem. However, none of these studies have examined the effects of background colour on cortisol levels of snapper. Inconsistent results have been reported in cortisol responses for the closely related red porgy *Pagrus pagrus* exposed to different background colours. Rotllant, Tort, Montero, Pavlidis, Martinez, Wendelaar Bonga and Balm (2003) observed higher levels in fish subjected to white backgrounds, while Van der Salm *et al.* (2004) noted no difference in cortisol levels following similar changes in background colour.

The aim of this study was to monitor changes in skin colour and plasma cortisol levels of snapper in response to a complete monochromatic change in background colour.

2. MATERIALS AND METHODS

2.1 Experimental procedures

A two-factorial experiment was conducted at the Port Stephens Fisheries Centre (PSFC) to investigate the rate of change in skin colour of live snapper transferred to different background colours [black to white (BW), or white to black (WB)] with time (0, 0.25, 0.5, 1, 2, 3, 5, 7 days). Each treatment comprised of three replicate cages, each containing four fish per cage. Fish were initially conditioned to two black or two white 1100 L tanks for 49 days while fed a commercial diet (50% protein, 12% fat; Barra Grower 18MJ, Ridley Agriproducts, Narangba, Queensland, Australia) supplemented with 39 mg astaxanthin kg^{-1} (Lucantin® Pink, BASF, Ludwigshafen, Germany) as recommended by Doolan, Booth, Allan and Jones (in press c [Chapter 4.4]). The skin colour of control fish [black to black (BB) and white to white (WW)] was evaluated at 0, 1 and 7 days. Plasma cortisol concentration was measured in two fish from each replicate for all treatments at 0, 1 and 7 days.

Snapper (437.4 ± 78.0 g; mean weight ± SD) were stocked into two black and two white 1100 L tanks at an initial density of ~25 kg m^{-3} and conditioned to their surroundings for 49 days. Fish were fed to apparent satiation twice daily (0.75–0.82 % biomass day^{-1}) at 09:00 and 15:00 hours (Australian Eastern Standard Time) and attained feed conversion ratios (FCRs) of 2.00–2.12 in black tanks and 2.25–2.32 in white tanks. The commercial diet was ground, mixed with astaxanthin and rebound as described by Doolan *et al.* (in press c [Chapter 4.4]). In addition, tanks were oxygenated during and immediately after feeding to maintain desirable dissolved oxygen levels. Measured water quality variables taken daily at 09:00 hours (Horiba U-10, Kyoto, Japan) were temperature (range 18.26–24.40°C), dissolved oxygen (>4.8 mg L^{-1}), pH (7.62–8.23) and salinity (32.16–33.28 g L^{-1}).

After 49 days, each tank of fish was anaesthetised (10 mg L^{-1} ethyl-*p*-aminobenzoate) and fish were transferred to a 200 L tub for further sedation (25 mg L^{-1} ethyl-*p*-aminobenzoate). Fish were randomly removed, measured for colour, weighed and stocked into white or black 200 L experiment cages. Black and white cages were randomly positioned around the perimeter of 10,000 L tanks (8 cages $tank^{-1}$) with individual inlets to each cage recirculating water at 2.5 L min^{-1} $cage^{-1}$. An additional three fish from each background colour conditioning tank (2 black tanks, 2 white tanks) were initially

sampled for plasma cortisol. At each sampling time thereafter, the 200 L tub described above was placed beneath designated cages, snapper sedated as described above (25 mg L^{-1} ethyl-*p*-aminobenzoate) and sampled. A previous pilot trial in which snapper were held in black or white cages and sampled under anaesthesia up to 5 min after disturbance indicated that this sampling protocol does not elevate plasma cortisol levels in snapper within the sampling time frame (B.J. Doolan, M.A. Booth, G.L. Allan and P.L. Jones, unpubl. data). Water temperature (22.06–24.12^{o}C), dissolved oxygen (>5.4 mg L^{-1}), pH (7.88–8.08), and salinity (31.90–32.15 g L^{-1}) were obtained daily at 09:00 hours. Snapper were not fed during this phase of the experiment.

2.2 Fish and facilities

Before experiments, PSFC produced Australian snapper were maintained in pale blue 10,000 L tanks within a sea-water recirculating system inside a shade house facility (Doolan, *et al.* 2007 [Chapter 4.2]) and fed a commercial barramundi *Lates calcarifer* diet (Barra Grower 18MJ; Ridley Agriproducts). Fish were starved for 24 h before all handling.

During the background colour conditioning phase of the experiment, fish were held in cylindrical black (L^* = 25.3, a^* = -0.3, b^* = 0.3; see below for method of measuring colour) or white (L^* = 61.4, a^* = -0.6, b^* = -1.5) polyethylene tanks (1.08 m diameter; 1.2 m depth; volume = 1100 L; Fylost Roto-Plastics, Brisbane, Queensland, Australia). During the transfer phase of the experiment fish were held in cylindrical cages (0.6 m diameter; 0.7 m depth; volume = 200 L) with walls lined with black (L^* = 26.0, a^* = -0.8, b^* = -0.2) or white (L^* = 88.4, a^* = -0.2, b^* = 1.1) plastic with bases made of 4 mm oyster mesh as described by Doolan, *et al.* (in press a [Chapter 4.3]). The cages were suspended within 10,000 L tanks. 10,000 and 1100 L tanks formed a recirculating system in which effluent water was channelled to two 500 L sumps, pumped through 15 µm cartridge filters to two 1300 L submerged biofilters and recirculated to tanks at approximately 20 L min^{-1}. Each tank was aerated with compressed air supplied through 4 x 100 mm diffusing airstones. System cartridge filters were exchanged daily and tanks vacuum siphoned twice weekly to remove accumulated solids.

2.3 Colour measurement

The colours of fish, tanks and cages were quantified by the CIE L^* a^* b^* colour scale using a handheld chroma-meter (CR-10, Minolta, UK; Selby Biolab, Gladesville, NSW, Australia). Anaesthetised fish were measured along three positions of the flank as described by Doolan, *et al.* (2007) (Chapter 4.2) and each colour value averaged to provide single L^* (black 0 to white 100), a^* (green -60 to grey 0 to red +60) and b^* (blue -60 to grey 0 to yellow +60) values for individual fish.

2.4 Blood sampling and analysis

Blood sampling was performed by inverting and holding live snapper within a foam sleeve lined with plastic. Approximately 2 mL blood was removed from the caudal vein with 23 G x 1.25 mm hypodermic needles and transferred to 2 mL tubes containing lithium heparin (Vacuette®, Greiner Bio-one, Australia). Blood samples were stored on ice before centrifugation at 6400 rpm (900 *g*) for 5 min. Plasma (approximately 1 ml) was removed and stored at -80^{o}C until assayed.

Plasma cortisol concentration was determined by radioimmunoassay. Plasma (100 µL) was extracted with 1 mL ethyl acetate, centrifuged at 3000 rpm (1200 *g*) for 5 min, and 100 µL aliquots evaporated to dryness. The residue was resuspended in 200 µL phosphate gelatine buffer, antibody and 200 µL ^{3}H steroid label and radioactivity counted. Extraction efficiency was 96.1% and all analyses were carried out in duplicate.

2.5 Statistical analyses

Data were assessed for homogeneity of variance in each case using Cochran's test before two-factor ANOVA. Where two-factor ANOVA revealed significant interactions (P<0.05), single-factor

ANOVA was conducted individually for each cage colour and for each time. The Student-Newman-Keuls (SNK) multiple comparison test was used to separate treatment means where ANOVA indicated significant differences ($P<0.05$). Where obvious asymptotes in colour values occurred, a break point time was estimated by the horizontal line and linear regression technique described by Sedgwick (1979). one-factor ANOVA was initially carried out independently for each cage colour treatment. Similarities ($P>0.05$) in L^* values between the latter sampling times for each background colour suggested a plateau in skin lightness and that maximum change in skin colour had occurred. An asymptote was derived from the mean of these values, and linear regression calculated through the preceding data points. Subsequently, the 'break point' was calculated from the intersection of the two lines indicating the optimal time of colour conditioning during the 7-day period. In other cases, data was modelled by non-linear regression. Statistical analyses were carried out using STATGRAPHICS PLUS, Version 4.1 (Manugistics, Rockville, MD, USA).

3. RESULTS

Snapper markedly changed skin lightness (L^*) following transfer from black to white (BW) or white to black (WB) backgrounds (Table 1, Figure 1) and there was a significant interaction between cage colour and time. Examining each cage colour level separately revealed no differences ($P>0.05$) between 0.25 and 5 days, and between 1 and 7 days in the BW fish after the initial rapid improvement in skin lightness (i.e. increased L^*), and between 0.5 and 7 days in the WB fish after the rapid initial darkening (i.e. reduced L^*) (Table 1). The break points estimated by intersecting line and linear regression indicated plateaus at $L^* = 65.2$ after 0.81 days (19 h) for fish moved from BW in comparison with $L^* = 54.6$ after 0.56 days (13 h) for WB fish (Figure 1). Substituting the same L^* plateaus into logarithmic functions revealed more conservative optimal times.

The change in L^* ($\Delta L^* = L^*_{final} - L^*_{initial}$ calculated from individual fish) predictably indicated a similar trend of rapid changes with no difference between 0.25 and 5 days in BW fish while a shift in time occurred in WB fish with no difference between 1 and 7 days (Table 1). There was a significant interaction between cage colour and time in L^* values of the control treatments (BB and WW). The skin colour of BB and WW controls remained greatly different at all times. There was an observable increase in skin lightness (L^*) of fish from WW controls ($P<0.05$) following the initial transfer from white tanks to white cages while BB controls barely changed.

Skin redness (a^* values) of BW fish continually declined over 7 days (Table 1, Figure 1). An initial insignificant decrease in a^* values also occurred in WW controls ($P = 0.06$). Conversely, a^* values of WB fish increased following transfer but did not continue to ascend in the manner of the decline observed in BW fish. a^* values of BB control fish remained constantly high.

There was no significant difference ($P>0.05$) in skin yellowness (b^* values) between cage colour treatments. Following an initial increase by 6 h, b^* values of BW fish decreased (Table 1, Figure 1). In contrast, overall b^* values of WB fish did not significantly differ ($P>0.05$) over the 7 days, however significant reductions occurred in the change in b^* (Δb^*) of individual fish beyond 1 day. No significant differences occurred in the change in b^* between cage colour treatments or between controls.

There was no effect of cage colour treatment (WB, BW), or the interaction between cage colour and time on plasma cortisol concentrations of snapper (Table 1). However, plasma cortisol levels were highest upon removal from black and white 1100 L tanks during the stocking of the experiment. In contrast, plasma cortisol levels of control fish (BB, WW) did not vary with time ($P>0.05$; Table 1). In addition, there were no significant effects of all colour treatments combined (ie. BW, WB, BB and WW) and time on plasma cortisol levels.

4. DISCUSSION

Transferring snapper to different background colours induced rapid changes in skin colour. The most marked change in skin lightness (L^*) occurred by the first sampling time at 6 h, reflecting physiological changes through the dispersal or aggregation of melanosomes within skin melanophores in response to the colour change in their surroundings (Fujii and Oshima, 1986; Fujii, 1993a). This supported previous findings (Doolan, *et al.* 2007 [Chapter 4.2]) of a striking contrast in the lightness of snapper taken from a black to white background following two days of transfer. The change in skin lightness from black to white or from white to black backgrounds closely mirrored one another suggesting physiological processes were reversed with a change in background colour.

Skin lightness in this study did not attain the levels of wild (Doolan, *et al.* 2007 [Chapter 4.2]) or previously reported cultured snapper (Doolan *et al.* in press a [Chapter 4.3]). One possibility for this difference is the addition of astaxanthin used during the current study. Astaxanthin increases redness (a^*) and this increased redness seems to reduce L^* values (i.e. darken; Doolan *et al.* in press a). While it is generally considered that melanophores affect L^* values, and skin erythrophores containing astaxanthin affect redness (a^*) or yellowness (b^*) values, L^* values decline with greater chroma in snapper (Doolan *et al.* in press a [Chapter 4.3]) and *P. pagrus* (Pavlidis, Papandroulakis & Divanach, 2006; Kalinowski, Izquierdo, Schuchardt & Robaina, 2007). However, because a^* values continued to decline in snapper transferred from black to white backgrounds during the seven day period, while L^* values became stable after the initial rapid change, we postulate that excessive numbers of melanophores were responsible for snapper not attaining their full potential lightness during this study.

Long-term conditioning of dark coloured snapper to white backgrounds can elevate skin lightness to the level of wild fish by morphological changes in melanophores (Doolan *et al.* in press a [Chapter 4.3]). Doolan *et al.* (in press a [Chapter 4.3]) observed greater numbers of melanophores and more intense melanin within skin melanophores in the skin of snapper 14 days after transferring from black to white cages in comparison with snapper produced in pale coloured surroundings and ongrown in white cages for 64 days. Therefore, a longer duration of exposure to white surroundings than the seven days of this study is needed to obtain maximal skin lightness via the degeneration of melanophores, similar to the observations of Sugimoto (1993) in *O. latipes*, rather than through the aggregation of melanosomes alone as we assume occurred during the current study. An entire production cycle in white surroundings may prevent the initial proliferation of melanophores. However, results here have shown an alternative for snapper growers producing snapper in sea cages involves holding fish within a white coloured conditioning facility for minimal time before sale. Less than one day was as effective in enhancing short-term skin lightness compared with periods up to seven days. The reverse darkening effect observed in snapper moved from black to white backgrounds in this study also reflects changes that may occur if hatchery reared snapper grown in white tanks are transferred to sea cages and shows that snapper will adapt skin colour rapidly to their new environment.

Interestingly, skin lightness of control snapper improved when transferred from the white tanks of the background colour conditioning period to white cages in the main experiment. It is possible that this observation was instigated by the reduction in stocking density (31 to 11 kg m^{-3}) and subsequent increase in visual contact of the fish with the surrounding walls. This may create stronger background adaptation in the skin colour of fish to the white walls to enable a greater level of skin lightness and is worthy of further investigation. The reverse was not apparent in the black control fish. Van der Salm, *et al.* (2004) similarly observed a darkening of *P. pagrus*, although insignificant, when fish were held at 25 kg m^{-3} in comparison with those held at 10 kg m^{-3}.

Skin redness (a^* values) of snapper transferred to white cages continually decreased with time (Figure 1). Although fish were deprived of astaxanthin throughout the seven day period, it is unlikely that astaxanthin would degrade or metabolise so quickly. Torrissen (1985) reported a decrease in flesh astaxanthin concentration following the cessation of feeding astaxanthin in rainbow trout *Salmo gairdneri*, however this was observed over months rather than days of depletion and was explained in part by flesh weight gain during this period. We speculate it is more probable that physiological

changes in erythrophores occurred, possibly to further conceal the appearance of the skin to the white background. Having said this, the sudden change in *L** values for fish transferred from black to white backgrounds was not accompanied by sudden changes in *a** or *b** values, suggesting that changes in the chromatic state of other skin chromatophores such as erythrophores or iridiphores may occur more gradually than changes in melanophores. Indeed, nervous influences are weaker on erythrophores (Fujii, 2000) and the rate of response of erythrophores is generally low compared to that of melanophores (Fujii, 1993a, b). However, as the change in redness occurred much more slowly than the change in lightness in this study, it is more likely that motile changes in melanophores are more crucial and preferentially used than those of erythrophores for stimulating rapid blending of snapper to white backgrounds. Such observations have not been previously made in sparids where erythrophores are present in high quantities (i.e. fish fed astaxanthin) in response to changes in background colour. We previously observed similar findings with lower skin redness observed after seven days when dark coloured snapper held in black cages were transferred to white cages while continually being fed astaxanthin supplemented diets (Doolan *et al.* in press a [Chapter 4.3]). Similarly, decreases in yellowness (*b**) occurred in snapper transferred from black to white cages in this study.

Predictably, the reverse transpired when snapper became redder (increased *a** values) as they became darker (increased *L**) when relocated from white to black backgrounds. However, skin yellowness decreased in not only fish moved from white to black backgrounds, but also in the black to black control fish. Reasons behind this are unclear and appear to be independent of background adaptation, in contrast with the more obvious cryptic colour changes previously explained in *L** and *a** values. As this study has only concentrated on live snapper, we are unsure whether these losses in colour will be restored to the skin post-mortem. Snapper may continue to lose redness with time following harvest and therefore, greater astaxanthin inclusion levels may be required in the diet to compensate for such changes.

Concentrations of plasma cortisol were notably highest when stocking fish from tanks into cages. The higher levels may be attributed to chronic stress instigated by the high densities (31 kg m^{-3}) at the end of the background colour conditioning phase. Similar findings have been reported with elevated cortisol in *P. pagrus* (Rotllant, Pavlidis, Kentouri, Abad & Tort, 1997; Rotllant & Tort, 1997; Rotllant, *et al.* 2003) and gilthead seabream *Sparus aurata* (Tort, Sunyer, Gómez & Molinero, 1996; Montero, Izquierdo, Tort, Robaina & Vergara, 1999) held at high densities (≥20 kg m^{-3}). While we did not sample snapper in the hours following transfer to cages, they may have displayed higher levels of cortisol immediately following the acute stress of transfer that decreased by our sampling at one day as reported by Maule, Tripp, Kaattari and Schreck (1989) in stressed chinook salmon *Oncorhynchus tshawytscha*. As plasma cortisol levels in snapper in the present study were not higher following transfer to different coloured cages, it is inferred that the fish acclimated quickly to their new surroundings and cage colour did not act as a chronic stressor upon the fish. Other authors have reported that subjecting fish to chronic stressors such as high density can lead to acclimation to the stressful conditions and a return to lower or basal levels (Pickering and Stewart, 1984) as displayed by Tort, *et al.* (1996) in the related *S. aurata*. In addition, it would appear that cortisol does not play a part in physiological colour changes of snapper in response to background colour as previously reported in *P. pagrus* (Szisch *et al.* 2002, Rotllant, *et al.* 2003; Van der Salm, *et al.* 2004; Fanouraki, Laitinen, Divanach & Pavlidis, 2007).

In conclusion, management practices that involve the transfer of dark coloured snapper to white tanks before live sale are highly beneficial and require less than one day. Such short-term changes in culture environment did not elicit a cortisol response in our snapper.

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TABLE 1

Measured (L^*, a^*, b^*) and changes (ΔL^*,Δa^*, Δb^*) in skin colour values, and plasma cortisol levels in snapper held or transferred to different background colours over 7 days. [1]

Colour	Time (days)	L^*	a^*	b^*	ΔL^*	Δa^*	Δb^*	Cortisol (ng ml^{-1})
Black-White	0	$52.6 \pm 0.7^{a\,x}$	$8.9 \pm 0.4^{c\,y}$	13.8 ± 0.1^{bcd}	0.0 ± 0.0^{a}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	38.8 ± 6.6^{b}
	0.25	64.2 ± 1.3^{bc}	7.5 ± 0.4^{bc}	15.2 ± 0.2^{d}	$12.1 \pm 1.8^{bc\,y}$	$-0.9 \pm 0.4^{b\,x}$	0.9 ± 0.4^{b}	-
	0.5	$62.2 \pm 1.1^{b\,y}$	7.6 ± 0.7^{bc}	14.7 ± 0.4^{d}	$8.5 \pm 1.0^{b\,y}$	-0.1 ± 0.6^{b}	0.8 ± 0.6^{b}	-
	1	$65.5 \pm 0.6^{bc\,y}$	7.0 ± 0.3^{ab}	15.0 ± 0.4^{d}	$11.6 \pm 2.4^{bc\,y}$	$-1.2 \pm 0.2^{b\,x}$	0.7 ± 0.7^{b}	8.5 ± 4.4^{a}
	2	$64.3 \pm 1.1^{bc\,y}$	$7.2 \pm 0.4^{ab\,x}$	14.5 ± 0.2^{cd}	$10.2 \pm 0.1^{b\,y}$	-0.9 ± 0.6^{b}	0.7 ± 0.4^{b}	-
	3	$64.1 \pm 0.4^{bc\,y}$	$6.6 \pm 0.2^{ab\,x}$	13.5 ± 0.3^{bc}	$10.7 \pm 0.7^{b\,y}$	$-1.8 \pm 0.3^{ab\,x}$	-0.6 ± 0.2^{ab}	-
	5	$64.5 \pm 0.7^{bc\,y}$	$6.4 \pm 0.4^{ab\,x}$	13.0 ± 0.5^{ab}	$13.0 \pm 0.6^{bc\,y}$	$-1.5 \pm 0.4^{ab\,x}$	-1.1 ± 0.3^{ab}	-
	7	$67.7 \pm 0.3^{c\,y}$	$5.5 \pm 0.3^{a\,x}$	12.2 ± 0.1^{a}	$16.3 \pm 0.6^{c\,y}$	$-2.8 \pm 0.1^{a\,x}$	-1.8 ± 0.5^{a}	21.7 ± 10.1^{ab}
White-Black	0	$65.6 \pm 0.9^{c\,y}$	$7.2 \pm 0.5^{a\,x}$	14.1 ± 0.2	0.0 ± 0.0^{c}	0.0 ± 0.0	0.0 ± 0.0^{cd}	37.2 ± 8.3^{b}
	0.25	59.2 ± 1.6^{b}	7.3 ± 0.5^{ab}	14.2 ± 0.5	$-7.0 \pm 1.0^{b\,x}$	0.5 ± 0.3^{y}	0.4 ± 0.1^{cd}	-
	0.5	$56.2 \pm 1.5^{ab\,x}$	7.4 ± 0.3^{ab}	14.3 ± 0.3	$-7.1 \pm 0.6^{b\,x}$	0.4 ± 0.4	0.0 ± 0.1^{d}	-
	1	$56.6 \pm 1.9^{ab\,x}$	8.1 ± 0.4^{ab}	14.2 ± 0.3	$-8.2 \pm 1.2^{ab\,x}$	1.0 ± 0.5^{y}	-0.7 ± 0.4^{abc}	23.9 ± 4.3^{a}
	2	$54.2 \pm 1.1^{a\,x}$	$8.9 \pm 0.1^{b\,y}$	14.8 ± 0.2	$-10.1 \pm 1.0^{ab\,x}$	0.8 ± 0.3	-0.1 ± 0.3^{cd}	-
	3	$53.8 \pm 0.6^{a\,x}$	$8.3 \pm 0.3^{ab\,y}$	14.2 ± 0.2	$-10.9 \pm 0.6^{ab\,x}$	1.4 ± 0.4^{y}	-0.4 ± 0.1^{bcd}	-
	5	$53.6 \pm 1.0^{a\,x}$	$8.5 \pm 0.3^{ab\,y}$	14.0 ± 0.4	$-11.1 \pm 1.3^{ab\,x}$	1.1 ± 0.7^{y}	-1.1 ± 0.2^{ab}	-
	7	$53.0 \pm 0.1^{a\,x}$	$7.9 \pm 0.1^{ab\,y}$	13.0 ± 0.5	$-12.5 \pm 0.8^{a\,x}$	0.6 ± 0.6^{y}	-1.5 ± 0.3^{a}	22.7 ± 8.8^{ab}
Controls								
Black-Black	0	52.6 ± 0.7^{x}	8.9 ± 0.4^{y}	13.8 ± 0.1^{b}	0.0 ± 0.0^{a}	0.0 ± 0.0^{y}	0.0 ± 0.0	38.8 ± 6.6
	1	52.5 ± 0.4^{x}	8.5 ± 0.2^{y}	14.7 ± 0.1^{c}	2.3 ± 0.5^{b}	0.3 ± 0.4^{y}	0.6 ± 0.4	15.4 ± 3.5
	7	52.1 ± 0.3^{x}	8.4 ± 0.5^{y}	12.8 ± 0.3^{a}	-0.9 ± 0.2^{a}	0.2 ± 0.7^{y}	-0.9 ± 0.1	36.4 ± 8.4
White-White	0	$65.6 \pm 0.9^{a\,y}$	7.2 ± 0.5^{x}	14.1 ± 0.2^{b}	0.0 ± 0.0	0.0 ± 0.0^{x}	0.0 ± 0.0	37.2 ± 8.3
	1	$67.3 \pm 0.3^{b\,y}$	6.1 ± 0.2^{x}	13.9 ± 0.2^{b}	1.4 ± 0.5	-0.9 ± 0.3^{x}	-0.6 ± 0.7	34.9 ± 11.7
	7	$68.7 \pm 1.1^{b\,y}$	6.0 ± 0.2^{x}	13.3 ± 0.2^{a}	3.9 ± 1.7	-0.7 ± 0.2^{x}	-1.1 ± 0.6	28.3 ± 9.3

[1] Values (means ± SEM) with the same letter in the superscript within each colour (between times; a, b) and within each time (between colours; x, y) are not significantly different (P>0.05, two-factor ANOVA, SNK). Where significant interactions occurred, values with the same letter in the superscript (in italics) within each colour (between times; *a, b, c, d*) and within each time (between colours; *x, y*) are not significantly different (P>0.05, one-factor ANOVA, SNK).

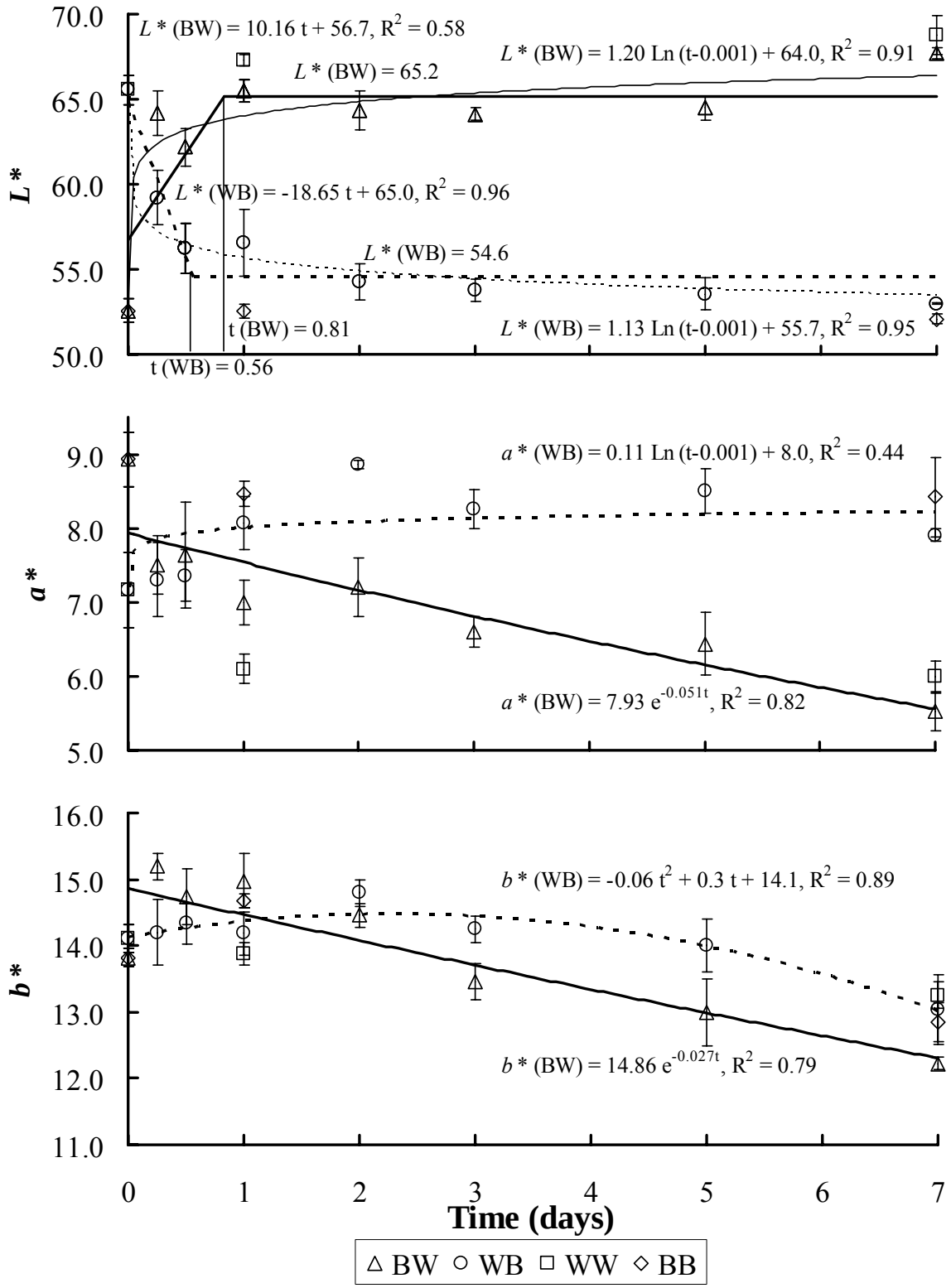

FIGURE 1
*L**, *a** and *b** values (means ± SEM) of snapper transferred from black to white (BW), white to black (WB), white to white (WW) or black to black (BB) backgrounds for 7 days

4.6 Effects of cage netting colour and density on the skin pigmentation and stress response of snapper *Pagrus auratus* (Bloch & Schneider, 1801)

Ben J. Doolan[1,2], Geoff L. Allan[2], Mark A. Booth[2], Paul L. Jones[1]

[1] *School of Life and Environmental Sciences, Deakin University, Warrnambool, Victoria 3280, Australia*
[2] *NSW Department of Primary Industries, Port Stephens Fisheries Centre and Aquafin CRC, Private Bag 1, Nelson Bay NSW 2315, Australia*

ABSTRACT

The unnaturally dark pigmentation of cultured Australian snapper *Pagrus auratus* can be improved through dietary astaxanthin supplementation and by holding fish in tanks with a white background. The practical application of these laboratory-based findings was examined with two experiments to establish if the advantages of transferring fish to light coloured tanks before harvest could be achieved on-farm using white cages and to determine the effects of fish density on skin colour. For the first experiment, snapper (mean TL = 29.7cm) were transferred from a commercial snapper sea cage to black or white netted cages and fed diets supplemented with unesterified astaxanthin (supplied as Lucantin® Pink, BASF) at 0 or 39 mg kg^{-1} for 42 days. Skin colour was measured using the CIE *L** (black – white), *a** (green – red), *b** (blue – yellow) colour scale. Snapper held in white netting cages became significantly lighter (higher *L**) than snapper held in black cages; however, values were not as high as previous laboratory-based studies in which snapper were held in white plastic-lined cages. Snapper fed astaxanthin displayed significantly greater *a** and *b** values, and total carotenoid concentrations after 42 days. In addition, total carotenoids were higher in fish from black than white cages. The second experiment was designed to investigate whether density reduced the improvements in skin colour achieved by holding fish in white coloured cages and whether cage colour affected stress. Snapper (mean weight = 435 g) were acclimated to black cages and fed 39 mg kg^{-1} astaxanthin for 44 days before transferring to black or white plastic-lined cages at 14 (low), 29 (mid) or 45 (high) kg m^{-3} for 7 days after which time skin colour, plasma cortisol and plasma glucose concentrations were measured. Skin lightness (*L**) was greater in snapper transferred to white plastic-lined cages with the lightest coloured fish obtained from the lowest density after 7 days. Density had no effect on plasma cortisol or glucose levels after 7 days, although plasma cortisol was elevated in snapper from black cages. For improved skin colouration we recommend feeding unesterified astaxanthin at 39 mg kg^{-1} for approximately 6 weeks and transferring snapper to white plastic-lined cages or similar at low densities for short periods before harvest rather than producing fish in white netting sea cages subject to biofouling.

1. INTRODUCTION

The skin colour of cultured sparids has received considerable attention in recent years. In particular, a deficiency of astaxanthin in standard commercial feeds has been implicated in the lack of pink colouration (Nakazoe, Ishii, Kamimoto & Takeuchi, 1984; Booth, Warner-Smith, Allan & Glencross, 2004; Chatzifotis, Pavlidis, Jimeno, Vardanis, Sterioti & Divanach, 2005; Kalinowski, Robaina, Fernández-Palacios, Schuchardt & Izquierdo, 2005; Doolan, Allan, Booth & Jones, in press a [Chapter 4.3]). In addition, the darkening caused by the excessive production of melanophores in Australian snapper *Pagrus auratus* (Booth, *et al.* 2004 [Chapter 4.1]; Doolan, Booth, Jones and Allan, 2007 [Chapter 4.2]), red sea bream *Pagrus. major* (Matsui, Tanabe, Furuichi, Yoshimatsu & Kitajima, 1992; Lin, Ushio, Ohshima, Yamanaka & Koizumi, 1998a, b) and red porgy *Pagrus pagrus* (Kolios, Kiritsis & Katribusas, 1997; Rotllant, Tort, Montero, Pavlidis, Martínez Wendelaar Bonga & Balm, 2003; Van der Salm, Martínez, Flik & Wendelaar Bonga, 2004) has been reported and negatively affects market value of cultured fish.

As sparids usually inhabit depths greater than those experienced in sea cages, a common culture practice is to shade cages to reduce incident lighting. While this can reduce skin darkness (Matsui,

et al. 1992; Coutteau, Robles, Ceulemansa & Van Halterena, 2002; Booth, *et al.* 2004 [Chapter 4.1]), even heavily shaded snapper do not lighten to the level of wild fish (Booth, *et al.* 2004 [Chapter 4.1]). More recent research has indicated that the most dominant factor influencing dark colouration in cultured snapper is the background colour of the culture environment (Doolan, *et al.* 2007 [Chapter 4.2]).

We have recently demonstrated that the skin colour of farmed snapper can be improved significantly by rearing fish at low densities in white-plastic lined cages while feeding 39 mg kg^{-1} unesterified astaxanthin for approximately six weeks in laboratory conditions (Doolan, Booth, Allan & Jones, in press b [Chapter 4.4]). However, this technology will be difficult to adopt for commercial sea cage operators who would have to transfer fish to land-based tanks to mimic the technology used. A much cheaper option would be to use cages with white netting. The effects of different coloured netting on the skin colour of snapper need to be measured and the practical problems with such a strategy assessed before it can be recommended for use with commercial sea cage*s*.

In addition, while it is possible to change (lighten) skin colour of snapper by holding them in white-coloured tanks before harvest, the effects of stocking density are unknown. As the skin colour changes Because of a camouflage response, it is likely that this will be mediated to some extent by the density of fish in the tank. There are limited studies available on density-influenced pigmentation in sparids. One such study by Van der Salm, *et al.* (2004) reported crowding of *P. pagrus* reduced skin lightness. However, the response was insignificant and only minor in comparison to the influential effect of tank colour.

The majority of research on density in aquaculture has focused on suppression of growth and immunodepression caused by chronic stress. While long-term secondary stress effects of hyperglycemia are common, chronic stress, such as that imposed by background colour or crowding, may also incur heightened plasma cortisol for many days or even weeks (Pickering & Pottinger, 1989), although typically below peak levels experienced from acute stress (Wendelaar Bonga, 1997). The high levels of cortisol and glucose observed by Montero, Izquierdo, Tort, Robaina and Vergara (1999) in gilthead seabream *Sparus aurata* after 15 weeks of crowding (40 kg m^{-3}) suggest that both cortisol and glucose may be suitable indicators of chronic stress imposed by background colour or crowding in snapper. The identification of such culture stressors is important as post-harvest flesh quality has been reported to deteriorate (increased *K*-value and onset of rigor mortis) in stressed snapper (Lowe, Ryder, Carragher & Wells, 1993).

Rotllant, *et al.* (2003) reported an increased incidence of stress in *P. pagrus* in white tanks, primarily displayed by increased plasma cortisol and glucose levels. Stressful stimuli are accompanied by rapid endocrine changes in the plasma concentrations of corticosteroids from the hypothalamic-pituitary-interrenal (HPI) axis and catecholamines from the hypothalamic-sympathetic-chromaffin cell axis (Wendelaar Bonga, 1997), known as primary effects of stress (Mazeaud, Mazeaud & Donaldson, 1977). These primary effects initiate secondary metabolic responses to deal with the increased energy demand such as elevated plasma glucose through increased glycogenolysis and/or gluconeogenesis (Gamperl, Vijayan & Boutilier, 1994). Hyperglycemia from acute stress is primarily initiated by glycogenolysis through catecholamine release (Sumpter, 1997; Wendelaar Bonga, 1997). Generally regarded as the main primary indicator of stress in teleosts, plasma cortisol, a glucocorticoid, is released from the interrenal cells of the head kidney (Van der Boon, Guido, Van den Thillart & Addink, 1991) for regulation of hydromineral balance (Wendelaar Bonga, 1997) and to mobilize energy reserves by gluconeogenesis (see reviews by Van der Boon *et al.* 1991; Wendelaar Bonga, 1997; Mommsen, Vijayan & Moon, 1999). Glucose elevations in response to chronic stress are more likely to be regulated by cortisol through the process of gluconeogenesis (Pottinger & Carrick, 1999) whereby non-carbohydrate substrates such as lipids and amino acids are used for glucose synthesis (Van der Boon, *et al.* 1991).

The aims of these experiments to enhance natural pigmentation in cultured snapper were to (i) determine the effects of cage (net) colour on skin pigmentation and (ii) evaluate the effects of density on the skin colour, and stress responses (cortisol – primary, and glucose – secondary) of snapper held in black and white cages.

2. MATERIALS AND METHODS

2.1 Experimental design and colour measurement

Two experiments were carried out. Experiment 1 was an orthogonal two-factor experiment designed to evaluate the interactive effects of cage colour (black or white net cages) and astaxanthin supplementation (0 or 39 mg kg^{-1}) on the skin colour of live snapper. Each treatment was replicated three times with 15 fish, and skin colour and total carotenoid concentrations were determined after 42 days. Experiment 2 was a two-factor experiment in which dark, red coloured snapper obtained from black cage adaptation and astaxanthin supplementation (39 mg kg^{-1}) for 44 days, were transferred to black or white plastic-lined cages at three densities (14, 29 or 45 kg m^{-3}) with four replicate cages for 7 days. Skin colour, cortisol and glucose levels were determined.

Skin colour was quantified using the CIE *L**, *a**, *b** colour scale where *L** represents lightness (0 = black to 100 = white), *a** represents green (-60) to red (+60) and *b** represents blue (-60) to yellow (+60). Single *L**, *a** and *b** values for fish were obtained by calculating the average of three measurements along the left flank of each fish (Doolan, *et al.* 2007 [Chapter 4.2]) using a handheld chroma-meter (CR-10, Minolta, UK; Selby Biolab, Gladesville, NSW, Australia).

2.2 Fish and facilities

Snapper of approximately 2.5 years of age used in experiments were progeny of first generation broodstock from the Port Stephens Fisheries Centre (PSFC). Fish in Experiment 1 were on-grown in sea cage*s* and fish in Experiment 2 were grown in a seawater recirculating system in pale blue tanks (*L** = 74.5, *a** = -14.7, *b** = 0.4) at PSFC. Handling protocols in all experiments included starving fish for 1 day before handling and anaesthesia with ethyl-*p*-aminobenzoate (10 mg L^{-1} light sedation, 25 mg L^{-1} heavy sedation).

Experiments were carried out at two locations. Experiment 1 was conducted at a commercial snapper farm [Silver Beach Aquaculture (SBA), Botany Bay, NSW, Australia, S 34°00′13″, E 151°12′26″]. The farm provided 36 m^2 (6 x 6 m; L x W) pontoons in which 125 m^3 sea cages or the experiment cages were positioned. Snapper for Experiment 1 were sourced from a 125 m^3 (5 x 5 x 5 m; L x W x H) black 15 mm net sea cage covered with white 'Sarlon' shade cloth (approximately 50% shade) to reduce algal growth and to exclude birds.

Experiment 2 was carried out at PSFC in two recirculating aquaculture systems inside a hothouse facility. System 1 consisted of two circular black (*L** = 27.9, *a** = -0.8, *b** = 1.7) 10,000 L tanks (3.4 x 1.1 m; *D* x *H*) in which experiment cages were positioned (4 cages $tank^{-1}$). Effluent water was mechanically and biologically filtered through a rotating drum filter fitted with a 40 µm screen (HYDROTECH, Vellinge, Sweden) and 3 x mixed bed bioreactors before returning through an oxygen saturator to tanks at a flow rate of approximately 80 L min^{-1}. System 2 consisted of 10,000 L tanks forming part of a recirculating system in which seawater was filtered to 20 µm through cartridge filters exchanged daily and through 2 x submerged biofilters. Water was pumped to each tank at approximately 20 L min^{-1}. Each tank was aerated with compressed air through 4 x 100 mm diffusing air stones and tanks vacuum siphoned twice weekly to dispose of accumulated solids and faecal matter.

Two varieties of experiment cages were used manufactured of netting or lined with plastic. 1 m^3 (1 x 1 x 1 m; *L* x *W* x *H*) black or white knotless mesh (9 mm black single layer, 15 mm white double layer) cages supported by PVC frames at top and bottom, were suspended from ropes within a

pontoon (12 cages pontoon^{-1}) at SBA or within tanks (4 cages tank^{-1}) of System 1 at PSFC. Cage bases were lined with black or white PVC sheeting to prevent inadvertent loss of feed. Individual cages were covered with 50 mm mesh securely fastened to prevent the escape of fish and the entire pontoon at SBA was covered with shade cloth as described above. Cylindrical floating 0.2 m^3 cages (0.6 x 0.7 m; D x H) were placed around the perimeter of 10,000 L tanks (8 cages tank^{-1}) in System 2 at PSFC. Cages formed a total surface area of 1.6 m^2 (1.32 m^2 walls, 0.28 m^2 base) with the walls lined with black (L^* = 25.6, a^* = -0.1, b^* = 1.2) or white (L^* = 88.1, a^* = 0.8, b^* = 4.9) PVC plastic and bases constructed of black or white 4 mm perforated mesh. Recirculated water was supplied to individual 0.2 m^3 cages at 2.5 L min^{-1}.

2.3 Diets

Snapper were fed commercial barramundi *Lates calcarifer* diets at SBA before Experiment 1 (45% protein, 20% fat; Skretting Pty. Ltd., Tasmania, Australia) and at PSFC before Experiment 2 (50% protein, 12% fat; Barra Grower 18MJ; Ridley Agriproducts, Narangba, Qld, Australia). Test diets for Experiments 1 and 2 were produced by re-milling the latter diet. Diets were thoroughly mixed with 50 g kg^{-1} 100% pre-gelatinised wheat starch (Pregel N, Penford Australia Ltd., Lane Cove, NSW, Australia) to assist with rebinding. The mashes were further mixed with or without 39 mg astaxanthin kg^{-1} (Lucantin® Pink, 10% formulation, BASF, Germany), and distilled water and pellets were extruded through a mincer fitted with a 6 mm pellet die (Barnco Australia Proprietary, Leichhardt, NSW, Australia). Diets were dried at <40^{o}C for approximately 3.5 h to obtain a slow sinking pellet of 7 – 9 % moisture and stored at <-15 ^{o}C until required.

2.4 Experiment 1. Effects of astaxanthin and cage netting colour on skin colour

Snapper (29.7 ± 2.1 cm; mean ± SD, n = 180) were removed from their sea cage holding pen at SBA, anaesthetized and individually measured for skin colour and length. Randomly selected fish were systematically allocated in groups of five fish to six black and six white cages, randomly positioned within the periphery of a 36 m^2 pontoon, until each cage contained 15 fish. An additional five fish were euthanized for carotenoid analysis. Fish were fed to apparent satiation twice daily at 09:00 and 14:00 hours with diets containing 0 or 39 mg astaxanthin kg^{-1} (LP39). All cages were replaced after 2 weeks but additional exchanges were not possible Because of prevailing strong winds. Water temperature ranged from 17.0 to 20.9^{o}C throughout the trial except for a 3-day period when it declined to 14.1^{o}C. After 42 days, cages were raised and fish promptly removed by net and transferred to oxygenated water containing 25 mg L^{-1} ethyl-*p*-aminobenzoate. The skin colour of all fish was recorded and three randomly selected fish from each cage were euthanized for carotenoid analysis.

2.5 Experiment 2. Effects of density on skin colour, plasma cortisol and plasma glucose

Snapper (630.1 ± 108.6 g; mean ± SD) with initial skin colour values (means ± SD, n = 32) of L^* = 65.0 ± 3.1, a^* = 5.5 ± 1.3, b^* = 12.7 ± 1.3, were acclimated for 44 days at an initial density of 18.9 ± 0.5 kg m^{-3} (mean ± SEM) in 8 x black 1 m^3 cages housed within black tanks of System 1 to provide a dark coloured environment. All fish were fed LP39 twice daily at 09:00 and 15:00 hours to apparent satiation. Water temperature (21.09 – 27.29 ^{o}C), pH (7.42 – 8.22), and dissolved oxygen (>5.7 mg L^{-1}) were measured daily with a handheld water quality analyser (Model 911, YeoKal Electronics, Brookvale, NSW, Australia). Growth and feeding variables (means ± SEM, n = 8 cages) during the acclimation period were weight gain (2.56 ± 0.12 g day^{-1}), feed consumption (0.63 ± 0.02 % biomass day^{-1}) and feed conversion ratio (1.72 ± 0.03).

After 44 days, snapper were transferred to 24 randomly positioned 0.2 m^3 cages (12 white, 12 black) in System 2 randomly assigned to three densities (low, mid, high) with four replicates per treatment. Water levels in 1 m^3 cages in System 1 were reduced to 0.5 m and fish lightly anaesthetised (10 mg L^{-1} ethyl-*p*-aminobenzoate). Cages of fish were transferred in sequence to a

separate container for further sedation (25 mg L^{-1} ethyl-*p*-aminobenzoate). Fish were randomly removed for colour measurements before systematically allocating in groups of four fish to 0.2 m^3 cages with four fish per low density (14.1 ± 0.4 kg m^{-3}), eight fish per mid density (29.4 ± 0.5 kg m^{-3}) and 12 fish per high density (44.7 ± 0.7 kg m^{-3}). Fish were fed to apparent satiation twice daily. Water temperature, pH, dissolved oxygen and salinity ranged from 21.10 – 22.70 ^oC, 7.66 – 8.26, 5.8 – 6.8 mg L^{-1} and 31.3 – 31.6 g L^{-1}, respectively. After 7 days, individual cages of fish were anaesthetized and colour measured. The first two fish removed from each cage were quickly measured for colour and blood immediately sampled (3 – 7 min post-disturbance).

2.6 Carotenoid determination

Snapper for carotenoid analysis were killed by seawater ice slurry, the left flank removed, vacuum packaged (-0.8 bar; Genius™, Vacuseal Pty. Ltd., Caboolture, Qld, Australia) and stored in the dark <-15^oC until required. Samples were slowly thawed and all flesh removed from the skin. The skin was homogenized and carotenoids extracted successively with 10 mL ethyl acetate:ethanol (1:1), 5 mL ethyl acetate, and 10 mL hexane as described by Doolan *et al.* (in press a [Chapter 4.3]). The supernatants were combined and evaporated to dryness before resuspending in 1 – 2 mL hexane containing 0.02% BHT (w/v). The absorbance was measured spectrophotometrically at 470 nm and total carotenoid concentration determined using $E_{1\%,1cm}$ = 2100 for astaxanthin in hexane.

2.7 Blood sampling and analysis

Anaesthetized snapper were inverted and restrained within a foam sleeve lined with moist plastic, and approximately 2 mL blood withdrawn by caudal puncture with 23 G x 1.25 mm hypodermic needles. Needle tips were removed and sub-samples of blood from each fish were evenly dispersed between two 2 mL tubes for cortisol (Vacuette®, Lithium heparin, Greiner Bio-one, Australia) and glucose (Vacuette®, FE Sodium Fluoride / EDTA K3, Greiner Bio-one, Australia) determination. Tubes were placed directly on ice and glucose samples immediately transferred to a NATA accredited laboratory for analysis. Plasma for cortisol analysis was obtained by centrifugation at 6400 rpm (900 *g*) for 5 min, carefully removed and stored at -80^oC until required for assay.

Plasma cortisol was measured by radioimmunoassay whereby 100 µL of plasma was extracted with 1 mL ethyl acetate, centrifuged for 5 min at 3000 rpm (1200 *g*) and duplicate 100 µL aliquots evaporated to dryness. The residue was resuspended in 200 µL phosphate gelatine buffer, antibody and 200 µL ^3H steroid label and radioactivity counted in a scintillation counter. Extraction efficiency was 99.6 %.

Plasma glucose was determined using the hexokinase enzymatic reference method described by Booth, Anderson and Allan (2006) on an automated analyzer (COBAS INTEGRA 700; Hunter Area Pathology Service, Newcastle, NSW, Australia).

2.8 Statistical analyses

Data were tested for homogeneity of variance by Cochran's test. When heterogeneous, $\log_{10}$ transformation restored homogeneity. Two-factor analysis of variance (ANOVA) was used to determine the effects of astaxanthin and cage colour on skin colour values and total carotenoid concentrations in Experiment 1, and to determine the effects of density and cage colour on skin colour, plasma cortisol and glucose levels in Experiment 2. The Student-Newman-Keuls (SNK) multiple comparison test was applied to separate treatment means where ANOVA indicated significant differences ($P < 0.05$). All data were statistically analysed using STATGRAPHICS PLUS, Version 4.1 (Manugistics, Rockville, MD, USA).

3. RESULTS

3.1 Experiment 1.

There were no significant interactions present between cage colour and diet for any skin colour values ($P > 0.05$). After 44 days, snapper were lighter (higher L^*) from white net cages than black net cages (Table 1). However, cage colour did not affect skin redness (a^*) or yellowness (b^*). In contrast, diet affected a^* and b^* with increased redness and yellowness ($P < 0.05$) attained in fish fed the LP39 diet. Similarly, total carotenoid content was greater when fish were fed LP39. In addition, fish from black cages displayed significantly greater total carotenoid concentrations. While fish growth was not recorded, there was no significant effect of cage colour or diet on daily feed consumption (7.3 ± 0.2 g fish^{-1} day^{-1}; grand mean ± SEM, $n = 12$).

3.2 Experiment 2.

Cage colour strongly affected L^* values ($P < 0.05$) of snapper 7 days following transfer from black surroundings (Table 2) with snapper held in white cages becoming lighter than those in black cages at all densities. Similarly, yellowness (b^*) was elevated in fish transferred to white cages. Significant interactions ($P < 0.05$) between cage colour and density in L^* and a^* values were primarily instigated by fish held at the low density in white cages. Low-density snapper were lighter and less red than fish at mid and high densities in white cages. Crowding of fish in black cages caused no measurable changes in skin colour.

Plasma glucose levels were similar ($P > 0.05$) between snapper from black and white cages (Table 2). However, plasma cortisol concentration was elevated ($P < 0.05$) in fish held in black cages. Plasma cortisol and glucose levels were not significantly affected by density although glucose was observably higher and more variable at the highest density in black and white cages.

4. DISCUSSION

Despite other authors reporting a reduction in the dark colouration of snapper and *P. major* by shading (Matsui, *et al.* 1992; Booth, *et al.* 2004 [Chapter 4.1]), the colour of cultured snapper held in sea cage*s* with 50% shading before Experiment 1 was dark. We found that wild snapper (mean TL = 25.0 cm, $n = 25$) and cultured snapper from pale blue coloured tanks of a recirculating system at PSFC (mean TL = 29.2 cm, $n = 16$), became much darker when transferred to the same shaded sea cages for approximately 4 months (mean ± SD; $L^* = 54.4 \pm 3.5$, $a^* = 6.8 \pm 2.1$, $b^* = 16.4 \pm 1.6$ for wild fish and $L^* = 50.0 \pm 4.9$, $a^* = 3.1 \pm 0.8$, $b^* = 11.9 \pm 1.4$ for cultured fish) (Doolan, *et al.* unpublished data). Employing white netting on cages during Experiment 1 improved skin lightness after 44 days but further enhancement was hindered by biofouling, especially during the final 4 weeks when cages could not be exchanged. The initial bright white appearance of the cages was tainted within several days, even under shaded conditions. We postulate that the most effective method to subject snapper to white surroundings for optimum skin colour may be to transfer fish to white tanks or plastic-lined cages on shore as previously suggested by Doolan, Booth, Allan and Jones (submitted [Chapter 4.5]).

Retaining dark-coloured snapper in white plastic-lined cages effectively improved skin lightness at all densities after 7 days. The lowest density used (14 kg m^{-3}, four fish 0.2 m^{-3}) which allowed increased contact of fish with the white surroundings ensured greatest skin lightness. Higher densities likely created a microenvironment whereby the dominant visual background became other fish rather than the colour of the cage. Using white tanks with large surface area to volume ratios would ensure greater contact of fish with the background and may enable greater numbers (i.e. higher densities) of fish to obtain maximum skin lightness through colour adaptation. The effect of large surface area to volume ratios by varying the area of walls or bases requires further investigation. The white bases used in the cages of Experiment 1 may have contributed to the improvement in skin lightness, similar to benthic dwelling fish such as flounder (Pleuronectiforms)

which lighten through adaptation to sandy substrates (Iwata & Kikuchi, 1998; Estevez, Kaneko, Seikai, Dores, Tagawa & Tanaka, 2001) or white tank bases (Yamanone, Amano & Takahashi, 2005), as the bases did not foul to the extent of the white netting. Lining the bases of sea cages to form a solid white substrate may be one such method to lighten snapper on site. Snapper held at the low density in white plastic-lined cages also displayed a reduced saturation of colour. This was associated with the improvement in skin lightness, contrary to the findings of Van der Salm, *et al.* (2004) who reported reduced chroma at higher density in *P. pagrus*. However, their diets did not contain astaxanthin. As previously reported by Doolan, *et al.* (submitted [Chapter 4.5]), snapper appear to alter the chromatic state of erythrophores (in the presence of astaxanthin) in addition to melanophores as a form of background adaptation when exposed to white surroundings.

Previous studies have reported resting plasma cortisol levels in wild snapper (Pankhurst & Sharples, 1992; Bollard, Pankhurst & Wells, 1993; Cleary, Pankhurst & Battaglene, 2000) and cultured *P. pagrus* (Rotllant, Pavlidis, Kentouri, Abad & Tort, 1997; Rotllant & Tort, 1997) are generally <10 ng mL^{-1} suggesting that there was no apparent enduring response of the HPI axis to crowding of snapper in black or white cages by 7 days in this study. Higher cortisol levels were obtained in black cages as reported in rainbow trout *Salmo gairdneri* (Gilham & Baker, 1985); however, remained within unstressed limits for the species. Although insignificant, plasma glucose levels were slightly higher and more variable at the highest density but similarly were low and in the range expected for unstressed snapper (<3 mM; Booth, *et al.* 2006), *P. pagrus* (Rotllant, *et al.* 2003; Van der Salm, *et al.* 2004) or *S. aurata* (Barton, *et al.* 2005).

Other authors have reported conflicting results in the response of *P. pagrus* to background colour or crowding stresses. Rotllant, *et al.* (1997) and Rotllant, *et al.* (2003) reported elevations in plasma cortisol after weeks of chronic crowding (20 kg m^{-3}), particularly in white tanks (Rotllant, *et al.* 2003). Both of these studies also observed the secondary effects of hyperglycemia after 9 days of crowding accompanying earlier peaks in plasma cortisol. Conversely, Van der Salm, *et al.* (2004) observed no differences in plasma cortisol or glucose concentrations of *P. pagrus* in response to tank colour (white, red or black) and crowding (10 or 25 kg m^{-3}) after 30 days, although cortisol levels were higher than our study and displayed greater variability, attributed to the extended handling time of their sampling protocol. Similarly, Rotllant and Tort (1997) and Van der Salm, Pavlidis, Flik and Wendelaar Bonga (2005) observed rapid elevations (within 10 min) of plasma cortisol in *P. pagrus* in reaction to acute stress. In contrast, sampling within 7 min of disturbance did not appear to elevate cortisol in this study or our previous study (Doolan, *et al.* submitted [Chapter 4.5]) and Pankhurst and Sharples (1992) did not observe elevations in cortisol of wild snapper until 30 min after disturbance.

The absence of primary and secondary stress effects in this study may be Because of a lower sensitivity to crowding or background colour in snapper than *P. pagrus*. Given that earlier sampling did not occur (<7 days) in this study, snapper may have reacted to the initial acute stressors of density and background colour with a transient change that quickly returned to baseline levels, as observed by Biswas, Seoka, Takii, Maita and Kumai (2006) with *P. major* in response to handling and confinement. Another possibility may be that the HPI axis was desensitized to potential stressors Because of the chronic stress of high density during the acclimation phase in black cages. While the average stocking density of 19 kg m^{-3} during the acclimation phase was moderate in the context of this study and in commercial snapper farming, it was considered high in the aforementioned crowding studies with other sparids. A suppression of the plasma cortisol response to acute stress after prolonged crowding has been observed in *S. aurata* (Barton, Ribas, Acerete & Tort 2005), and in *P. pagrus* pre-exposed to black tanks with no such adaptation observed in white tanks (Rotllant, *et al.* 2003). Despite these presumptions, snapper in this study were not stressed based on these parameters in either white or black cages at all densities after 7 days.

Supplementing unesterified astaxanthin at 39 mg kg^{-1} (LP39) in feeds for >42 days was effective in improving skin redness and yellowness in Experiments 1 and 2 as recommended by Doolan *et al.* (in press b [Chapter 4.4]). Although feed consumption was similar and there were no observable

differences in fish feeding behaviour between cage colours, the higher total carotenoid concentrations observed in the black netting cages of Experiment 1 complement previous findings of Doolan, *et al.* (in press a [Chapter 4.3]) in which black cage adapted snapper exhibit higher carotenoid levels after feeding astaxanthin. While significantly higher carotenoid concentrations were observed in fish in black cages without astaxanthin supplementation, levels in black and white cages were both low and similar in magnitude to other studies on snapper (Booth, *et al.* 2004 [Chapter 4.1]; Doolan, *et al.* in press a [Chapter 4.3]) and the difference may be attributed to the loss of one replicate cage of fish causing a loss of power from the design.

In conclusion, to improve skin lightness without elevating plasma cortisol or glucose concentrations, snapper should be held within solid walled white surroundings such as white tanks for short periods rather than long-term growing in white netting sea cages. While holding snapper in white cages at high densities greatly improves skin lightness in comparison with black cages, it is recommended that snapper be held at low densities to further enhance skin lightness. In addition, snapper should be fed 39 mg unesterified astaxanthin kg^{-1} for 6 weeks to enhance pink pigmentation before harvest.

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TABLE 1
*L**, *a** and *b** skin colour values, and total carotenoid concentrations of snapper held in black or white netting cages and fed 0 or 39 mg kg^{-1} astaxanthin (LP39) (Experiment 1).[1,2]

Colour	Diet	*L**	*a**	*b**	Total carotenoids (µg g^{-1})
Black	0	50.3 ± 1.3 ^{x}	5.3 ± 1.2 ^{a}	7.1 ± 0.4 ^{a}	2.90 ± 0.23 $^{a\ x}$
	LP39	48.4 ± 1.6 ^{x}	8.5 ± 0.9 ^{b}	8.8 ± 0.4 ^{b}	7.72 ± 0.73 $^{b\ x}$
White	0 [3]	56.6 ± 0.1 ^{y}	5.8 ± 0.3 ^{a}	7.1 ± 0.8 ^{a}	2.18 ± 0.14 $^{a\ y}$
	LP39	56.3 ± 0.3 ^{y}	7.0 ± 0.5 ^{b}	9.5 ± 0.6 ^{b}	5.95 ± 0.30 $^{b\ y}$

[1] Values (mean ± SEM, $n = 3$) with different letters in the superscript within each colour (between diets; a, b) and within each diet (between colours; x, y) are significantly different ($P < 0.05$; two-factor ANOVA, SNK).
[2] Initial values (mean ± SD) of snapper were *L** = 54.4 ± 4.4, *a** = 5.8 ± 1.7, *b** = 9.0 ± 1.8, total carotenoids (470 nm) = 2.66 ± 0.31 µg g^{-1}
[3] $n = 2$ Because of the loss of one replicate cage of fish

TABLE 2
*L**, *a** and *b** skin colour values, plasma cortisol and glucose levels of snapper held in black or white plastic-lined cages at high or low densities after 7 days (Experiment 2). [1,2]

Colour	Density	*L**	*a**	*b**	Cortisol (ng ml^{-1})	Glucose (mM) [3]
Black	Low	50.7 ± 1.6 *x*	8.2 ± 0.3	9.3 ± 0.4 x	9.9 ± 5.0 y	0.95 ± 0.15
	Mid	51.4 ± 0.9 *x*	8.0 ± 0.2	9.2 ± 0.2 x	10.0 ± 3.4 y	0.49 ± 0.11
	High	50.8 ± 1.3 *x*	7.8 ± 0.1 *x*	9.8 ± 0.3 x	10.2 ± 2.7 y	1.25 ± 0.66
White	Low	60.4 ± 0.9 *b y*	7.5 ± 0.3 *a*	10.6 ± 0.2 y	2.1 ± 1.6 x	0.49 ± 0.09
	Mid	57.4 ± 0.5 *a y*	8.6 ± 0.2 *b*	10.6 ± 0.3 y	3.1 ± 1.7 x	0.54 ± 0.13
	High	57.4 ± 1.0 *a y*	9.1 ± 0.2 *b y*	10.4 ± 0.2 y	3.9 ± 2.8 x	1.05 ± 0.41

1 Values (mean ± SEM) with different letters in the superscript within each density (between colours; x, y) are significantly different ($P < 0.05$; two-factor ANOVA, SNK). Where significant interactions occurred, values with different letters in the superscript (in italics) within each colour (between densities; *a, b*) and within each density (between colours; *x, y*) are significantly different ($P < 0.05$; one-factor ANOVA, SNK).
2 Initial values (mean ± SD) of snapper following acclimation to black surroundings were *L** = 49.0 ± 3.2, *a** = 8.7 ± 1.4, *b** = 10.8 ± 1.2.
3 Data $\log_{10}$ transformed to homogenize variances

4.7 Effects of cage colour and post-harvest K^+ concentration on skin colour of Australian snapper, *Pagrus auratus* (Bloch & Schneider, 1801)

Ben J. Doolan[1,2], Mark A. Booth[2], Geoff L. Allan[2] and Paul L. Jones[1]

[1] *School of Life and Environmental Sciences, Deakin University, Warrnambool, Victoria 3280, Australia*
[2] *NSW Department of Primary Industries, Port Stephens Fisheries Centre and Aquafin CRC, Private Bag 1, Nelson Bay NSW 2315, Australia*

ABSTRACT

In an attempt to improve post-harvest skin colour in cultured Australian snapper *Pagrus auratus*, a two-factor experiment was carried out to investigate the effects of a short-term change in cage colour before harvest followed by immersion in K^+-enriched solutions of different concentrations. Snapper supplemented with 39 mg unesterified astaxanthin kg^{-1} for 50 days were transferred to black (for 1 day) or white cages (for 1 or 7 days) before euthanasia by immersing fish in seawater ice slurries supplemented with 0, 150, 300, 450 or 600 mmol L^{-1} K^+ for 1 h. Each treatment was replicated with five snapper (mean weight = 838 g) held individually within 0.2 m3 cages. *L**, *a** and *b** skin colour values of all fish were measured after removal from K^+ solutions at 0, 3, 6, 12, 24 and 48 h. After immersion in K^+ solutions, fish were stored on ice. Both cage colour and K^+ concentration significantly affected post-harvest skin colour ($P < 0.05$), and there was no interaction between these factors at any of the measurement times ($P > 0.05$). Conditioning dark coloured snapper in white surroundings for 1 day was sufficient to significantly improve skin lightness (*L**) after death. Although there was no difference between skin lightness values for fish held for either 1 or 7 days in white cages at measurement times up to 12 h, fish held in white cages for 7 days had significantly higher *L** values (i.e. they were lighter) after 24 and 48 h of storage on ice than those held only in white cages for 1 day. K^+ treatment also affected (improved) skin lightness post harvest although not until 24 and 48 h after removal of fish from solutions. Before this time, K^+ treatment had no effect on skin lightness. Snapper killed by seawater ice slurry darkened (lower *L**) markedly during the first 3 h of storage in contrast with all K^+ treatments which prevented darkening. After 24 and 48 h of storage on ice, fish exposed to 450 and 600 mmol L-1 K^+ were significantly lighter than fish from seawater ice slurries. In addition, skin redness (*a**) and yellowness (*b**) were strongly dependent on K+ concentration. The initial decline in response to K^+ was overcome by a return of *a** and *b** values with time, most likely instigated from a re-dispersal of erythrosomes in skin erythrophores. Fish killed with 0 mmol L^{-1} K^+ maintained the highest *a** and *b** values after death, but were associated with darker (lower *L**) skin colouration. It is concluded that a combination of conditioning snapper in white surroundings for 1 day before harvest followed by immersion in seawater ice slurries supplemented with 300 to 450 mmol L^{-1} K^+ improves skin pigmentation after >24 h of storage on ice.

1. INTRODUCTION

Despite declines in commercial catches and the popularity of Australian snapper *Pagrus auratus* as a table fish, the unnatural skin pigmentation of cultured snapper is a problem affecting the viability of sea cage snapper farming throughout Australia. Farmed snapper generally lack the pinkness of wild fish when astaxanthin is not supplemented in formulated diets (Booth, Warner-Smith, Allan & Glencross, 2004 [Chapter 4.1]; Doolan, Allan, Booth & Jones, in press a [Chapter 4.3]); however, the darkness of snapper produced in sea cages in particular is viewed as a major factor governing inferior consumer acceptance and market value (Doolan, Booth, Jones & Allan, 2007 [Chapter 4.2]).

The skin colour of teleosts is Because of the existence of chromatophores, capable of varying expression by physiological or morphological changes (see reviews by Fujii & Oshima, 1986;

Fujii, 1993a). The chromatophores primarily responsible for the darkness and redness of snapper and other sparids are melanophores and erythrophores. Motile actions of chromatophores are controlled by the sympathetic nervous and endocrine systems (Fujii & Oshima, 1986; Oshima, Suzuki, Yamaji & Fujii, 1988; Fujii, 1993b), with sudden changes in the chromatic state instigated by neural excitation. In particular, rapid blanching of the skin is adrenergic, with norepinephrine being the main neurotransmitter (Kumazawa & Fujii, 1984). The melanosome-aggregating nerve terminals release the transmitter which acts on α-adrenoceptors on the melanophore membrane to instigate melanosome aggregation (Oshima, *et al.* 1988). Elevated levels of K^+ activate neuron termini to release norepinephrine, and for this reason has been artificially applied to improve or maintain post-mortem skin lightness by aggregating melanosomes within melanophores in red sea bream *Pagrus major* (Lin, Ushio, Ohshima, Yamanaka & Koizumi, 1998a; b). In addition, K^+ can induce blanching of erythrophores and initiate a subsequent loss of redness via norepinephrine release on α-adrenoceptors (Matsumoto, Watanabe & Obika, 1978; Karlsson, Anderson, Elwing & Grundstrom, 1988; Lin, *et al.* 1998b) or directly by depolarization of erythrophores in some species (Karlsson, *et al.* 1988), in contrast with the adrenergic response only of melanophores (Fujii, 1993a). Despite the positive effects of K^+ on melanophores, K^+ can have a negative impact by reducing skin redness (Lin, *et al.* 1998b). Owing to the adrenergic response of melanophores, cells must be innervated for K^+ to take effect, and its efficacy has been reported up to 30 min after death in *P. major* (Lin, *et al.* 1998b).

Changing the culture conditions in combination with prior dietary astaxanthin supplementation has led to improved skin pigmentation in live snapper. While long-term exposure to white surroundings is recommended for maximal skin lightness (Doolan, *et al* in press a [Chapter 4.3]), improved skin lightness (during 7 days of observations) has been achieved in dark-coloured snapper within 1 day of transfer to white cages through background adaptation (Doolan, Booth, Allan & Jones, submitted [Chapter 4.5]). While these findings may prove beneficial for those targeting the live fish markets, it has yet to be shown whether these improvements can be maintained in cultured snapper following death. Some authors have reported a darkening of the skin of *P. major* after death (Lin, *et al.* 1998a, b) masking the beneficial 'wild-like' appearance displayed when alive. Generally, the slaughter technique practiced by commercial snapper operators to obtain the best colour and appearance is by ice-slurry immersion to rapidly chill core temperature, followed by packing in plastic surrounded by ice (Harada, 1988; Connell, 1990). Lin, *et al.* (1998b) found that immersing *P. major* in K^+ solutions for extended periods (e.g. 150 h) improved skin colour, but there is no information on the effect of short-term immersion in K^+ solutions on skin colour after different periods of storage.

As such, the aim of this experiment was to examine the interactive effects of pre-harvest cage colour conditioning and short-term immersion in K^+ enriched seawater ice slurries on post-harvest skin colour attributes of cultured Australian snapper.

2. MATERIALS AND METHODS

2.1 Experimental design and measurement of skin colour

A two-factor experiment was conducted to determine the effects of pre-harvest conditioning in white or black cages and post-harvest immersion in K^+ solutions of different concentrations on the skin colour of snapper after slaughter and storage for different periods of time up to 48 h. The study included three phases summarized in Table 1: (1) pre-experiment phase, (2) conditioning phase and (3) K^+ experimental phase. Snapper were held in black cages for 150 days and supplied with 39 mg kg^{-1} unesterified astaxanthin to induce dark, red skin pigmentation (Doolan, *et al.* submitted [Chapter 4.5]); Doolan, Booth, Allan and Jones, in press b [Chapter 4.4]). Fish were transferred to individual cages comprising of three pre-harvest conditioning treatments: 1 day in white (W1), 1 day in black (B1), or 7 days in white (W7) cages. Snapper were then slaughtered by seawater ice-slurry immersion and soaked for 1 h before storage on ice. KCl was added to the ice slurries to give 0, 150, 300, 450 or 600 mmol L^{-1} K^+. There were five replicate cages/fish for each treatment

combination giving 75 cages in total. Skin colour was measured immediately (t = 0) and at 3, 6, 12, 24 and 48 h after removal from the slurries using a hand-held chroma-meter (CR-10, Minolta, UK: Selby Biolab, Gladesville, NSW, Australia).

Colour measurements were taken from three positions along the left flank of fish and averaged as described by Booth *et al.* (2004 [Chapter 4.1]) to obtain representative L^* (lightness = black 0 to white 100), a^* (green -60 to red +60), and b^* (blue -60 to yellow +60) values for each fish. The colours of tanks and cages were similarly measured. Although presented by other authors (Van der Salm, Martĭnez, Flikand & Wendelaar Bonga, 2004; Chatzifotis, Pavlidis, Jimeno, Vardanis, Sterioti & Divanach, 2005; Kalinowski, Robaina, Fernández-Palacios, Schuchardt & Izquierdo, 2005), hue [H_{ab} = arctan (b^* / a^*)] was not derived from a^* and b^* values as minor changes in magnitude when close to achromatic (a^* = 0, b^* = 0), as found with snapper exposed to greater K^+ concentrations, lead to substantial irrelevant changes in the hue angle (eg. a^* = 0.9, b^* = 0.1, H_{ab} = 6°; a^* = 0.1, b^* = 0.9, H_{ab} = 83°; a^* = -0.9, b^* = 0.1, H_{ab} = 173°; a^* = 0.1, b^* = -0.9, H_{ab} = 276°).

2.2 Fish and facilities

Before this study, snapper of approximately 2.5 – 3 years of age were fed a commercial barramundi feed (Barra Grower 18 MJ, 50% protein, 12% fat; Ridley Agriproducts, Narangba, Qld, Australia) twice daily and grown in pale blue (L^* = 74.1, a^* = -12.2, b^* = 4.2) tanks in a seawater recirculating system at the Port Stephens Fisheries Centre (PSFC). The system (System 1) contained 2 x 10,000 L tanks (3.4 m diameter, 1.1 m depth) housed within a greenhouse facility. Effluent water passed through a sludge collector to a rotating drum filter or was filtered directly through the rotating drum filter fitted with a 40 μm screen (HYDROTECH, Vellinge, Sweden) providing approximately 5% exchange of system water daily, to 3 x mixed bed bio-reactors. Recirculated water was pumped through an oxygen saturator to each tank at approximately 80 L min^{-1}.

Experiment phases 1 and 2 were carried out within a second recirculating system (System 2) consisting of 10,000 L tanks housed within the greenhouse of System 1. Particle filtration was provided by 20 μm cartridge filters and biofiltration through 2 x submerged biofilters. Water recirculated to tanks at approximately 20 L min^{-1}, and each tank was supplied with compressed air through 4 x 100 mm diffusing air stones. Cartridge filters were exchanged daily and tanks vacuum siphoned twice weekly to dispose of accumulated solids and faecal matter. Water temperature was maintained by 1 x 2 kW immersion heater in each tank.

Two cage designs were used in the experiment. During the pre-experiment phase (Phase 1) the walls and base of a 1 x 10,000 L tank were lined with black (L^* = 28.5, a^* = -0.8, b^* = -1.4) plastic to provide entirely black surroundings and 4 x 1 m^3 (1 m x 1 m x 1 m; L x W x H) cages constructed of black 9 mm knotless mesh were suspended within the tank. During the conditioning phase (Phase 2) cylindrical floating 0.2 m^3 cages (0.6 m diameter, 0.7 m depth) were suspended around the perimeter of 10 x 10,000 L tanks (8 cages per tank). Cages were lined with black (L^* = 25.4, a^* = -1.0, b^* = 0.3) or white (L^* = 89.1, a^* = -1.0, b^* = 0.4) PVC plastic.

During handling procedures in Phases 1 and 2, snapper were anaesthetized *in situ* with ethyl-*p*-aminobenzoate (10 mg L^{-1}) and transferred to a black oxygenated tub for further sedation (25 mg L^{-1}).

2.3 Experimental procedures

Snapper were held in black cages suspended within a black tank at moderate densities for 150 days during the pre-experiment phase. Fish were fed to apparent satiation once daily with a commercial barramundi feed (Barra Grower 18MJ, 50% protein, 12% fat; Ridley Agriproducts) containing 39 mg kg^{-1} astaxanthin (Lucantin® Pink, BASF, Ludwigshafen, Germany) as recommended by Doolan

et al. (in press b) [Chapter 4.4] for the final 50 days. Fish consumed 0.45 ± 0.01 % BW day^{-1} (mean ± SEM, n = 4 cages).

For the conditioning phase, 75 x 0.2 m^3 (50 white, 25 black) cages allocated between each conditioning treatment (W1, B1, W7) were randomly positioned throughout the 7 x 10,000 L tanks. Lightly anaesthetized snapper were randomly removed from 1 m^3 pre-experiment cages, further sedated and weighed before they were systematically allocated to 0.2 m^3 cages. Each cage was stocked with a single fish to ensure independent sampling during the following K^+ experimental phase. Fish were exposed to a 24:0 h (L:D) photoperiod and starved during the conditioning period.

K^+ solutions for the K^+ experimental phase were prepared by dissolving potassium chloride in seawater (335 mmol L^{-1} Na^+, 8 mmol L^{-1} K^+, 7 mmol L^{-1} Ca^{2+}, 27 mmol L^{-1} Mg^{2+}) at room temperature to obtain the required K^+ concentration. Concentrations of other cations were not altered to enable simple transfer to commercial applications. To minimise dilution of K^+ concentrations caused by the melting of ice in slurries, stock solutions for each concentration were refrigerated to 1 - 3^{o}C before use. Individual slurries for each cage were prepared within 10 L buckets containing approximately 7 L of the stock solutions and made up to volume with ice. Stock concentrations of 0, 150, 300, 450 and 600 mmol L^{-1} K^+ solutions were determined using flame atomic absorption (Hunter Water Laboratories, Hunter Water Australia, Warabrook, NSW, Australia). Analysed concentrations were 8, 153, 314, 468 and 618 mmol K^+ L^{-1}, respectively, upon immersion of fish and 12, 130, 251, 403 and 481 mmol K^+ L^{-1} after 1 h. After the required conditioning time (1 or 7 days) in 0.2 m^3 cages, fish were rapidly removed by a net and immediately submerged in their individual K^+ slurries. Cages were randomly selected and fish were removed 45 s apart to ensure manageability of latter colour measurements. Fish were removed from slurries precisely 1 h after immersion, measured for colour *(*t = 0 h), individually wrapped in plastic and packed surrounded by ice inside foam boxes (0^{o}C). Subsequent measurements were taken 3, 6, 12, 24 and 48 h after removal from slurries.

2.4 Statistical analyses

All statistical tests were conducted using STATGRAPHICS PLUS, Version 4.1 (Manugistics, Rockville, MD, USA). All data were tested for homogeneity of variance (Cochran's test) and normality, and arcsin transformed when heterogeneous or skewed. One extreme outlier from the W7 150 mmol L^{-1} treatment with exceptionally high L^* values at all storage times was omitted from analyses. Two-factor analysis of variance (ANOVA) was applied separately at each storage time on L^*, a^* and b^* colour values. Where ANOVA was significant ($P < 0.05$), the Student-Newman-Keuls (SNK) multiple comparison test was used to separate treatment means.

3. RESULTS

While conditioning treatment and soaking in K^+ solutions affected skin values of snapper, there were no significant interactions ($P > 0.05$) between these factors in L^*, a^* or b^* values at any of the times measured.

3.1 Cage colour

Snapper transferred to black cages for 1 day (B1) were always darker than fish transferred to white cages (W1 and W7) at all K^+ concentrations over all times sampled (Figure 1). L^* values reduced for B1 fish (i.e. skin colour darkened) between 0 and 3 h storage on ice but did not darken thereafter. The L^* values for fish conditioned in white cages for 1 or 7 days (W1 or W7) before harvest were similar for 0, 3, 6 and 12 h post harvest, but after this time the L^* values remained constant for fish previously held in white cages for 1 day (W1), while L^* values increased for fish held in white cages for 7 days (W7) (Figure 1). Both a^* (Figure 2) and b^* (Figure 3) values of snapper were similar ($P > 0.05$) from all conditioning treatments (B1, W1, W7) upon removal from K^+ solutions (0 h). a^* and b^* values of all fish were similar or decreased from 0 to 3 h before

increasing after 12 h. The *a** and *b** values of W7 conditioned snapper remained lower than the W1 and B1 fish from 3 h onwards.

3.2 K^+ solutions

Skin lightness (*L**) was not affected ($P > 0.05$) by K^+ concentration for measurements at 0, 3, 6 or 12 h of storage on ice (Figure 1). Conversely, K^+ concentration affected skin *L** after 24 and 48 h. After 24 h, the *L** value for fish not exposed to K^+ (seawater ice slurry, 0 mmol L^{-1}) at harvest reduced (i.e. fish became darker) and fish soaked in 450 and 600 mmol L^{-1} K^+ were significantly lighter. Although the nape of fish was not measured, it was noticeably darker for white cage-conditioned fish (W1 and W7) not exposed to K^+, especially in comparison with fish soaked in >300 mmol L^{-1} K^+. The nape of all B1 fish was dark regardless of K^+ concentration.

Soaking in K^+ solutions strongly reduced *a** and *b** values (Figures 2 and 3). Fish killed in a seawater ice slurry (0 mmol L^{-1}) maintained higher *a** and *b** values from removal to 48-h storage. For the first 6 h of storage, there was no difference in redness (*a**) between 150 and 600 mmol L^{-1} K^+ (Figure 2). Thereafter, *a** returned to some extent to the skin and was notably more rapid in fish exposed to lower concentrations. By 48 h, there were no significant differences in *a** beyond 300 mmol L^{-1}. Initially, a strong response to K^+ was evident in *b** whereby values declined with increasing concentration until 6-h storage (Figure 3). In general, *b** increased in magnitude thereafter, and by 48 h there were no significant differences beyond 300 mmol L^{-1} as observed in the case of *a**.

4. DISCUSSION

Conditioning dark-coloured snapper in white cages before harvest was an effective means of improving skin lightness after death. As melanophore degradation typically transpires from long-term exposure to background colour (Sugimoto, 1993), it is unlikely that snapper decreased the net melanin content of the skin within 1 day, however melanin concentrations were not measured in this study. Even so, 1 day was sufficient to sustain skin lightness after death, most likely stimulated by neuro-endocrine regulated aggregation of melanosomes within melanophores (Fujii and Oshima, 1986). After storage on ice for 24 h, fish previously conditioned in white cages for 7 days were significantly lighter than fish conditioned in white cages for 1 day, which may have been Because of a reduction in the melanin content. For commercial purposes, when a rapid turnover of fish is required, transferring snapper to white surroundings for 1 day may prove most cost-effective in reducing skin darkness. In addition, conditioning in white cages for 7 days also strongly reduced chroma (*a** and *b** values) after death as observed previously in live snapper by Doolan, *et al.* (submitted) [Chapter 4.5]. This may have been a persistent adaptation to camouflage to the white surroundings by aggregating erythrophores, but it was not considered to be favourable, as a higher intensity of the red pigmentation is generally a market advantage (Doolan, *et al.* 2007 [Chapter 4.2]).

The post-mortem darkening common in cultured sparids (Lin, *et al.* 1998a, b), was evident in snapper killed in seawater ice slurries. Lin, *et al.* (1998b) suggested that such darkening from melanophore motility is possibly caused by handling and killing stresses. As wild snapper do not exhibit the proliferation of melanophores of farmed snapper, skin darkening through melanosome dispersal Because of these harvest stresses is likely to be limited and reduces the need for using treatments such as those of this study when harvesting wild snapper. In the cultured snapper of this study, soaking in K^+ solutions prevented the initial darkening during the first 3 h of storage on ice. Most importantly, greater skin lightness was achieved after prolonged storage in fish soaked in 450 – 600 mmol L^{-1} K^+. Interestingly, fish exposed to higher K^+ concentrations were darker after removal from slurries (1 h post immersion) in this study, despite a rapid melanosome aggregation (10 min) observed in *P. major* soaked in 480 mmol L^{-1} (Lin, *et al.* 1998b). However, following soaking in K^+ solution and storage on ice, fish from these treatments lightened. The stability in skin lightness after prolonged storage at 0^oC suggests that 1-h immersion is suitable for snapper.

Correspondingly, storage at a low temperature enhanced K^+ induced melanosome aggregation in *P. major* (Lin, *et al.* 1998a). Although Lin, *et al.* (1998b) reported that immersion in K^+ solutions was effective up to 30 min after death, incorporating KCl into ice slurries minimizes handling time and is an effective method with this species.

While the main purpose of K^+ was to reduce dark colouration by acting upon melanophores, soaking in K^+ solutions also mediated changes in red colouration as described by others (Karlsson, *et al.* 1988; Lin, *et al.* 1998b; Lin, *et al.* 1998c). It appeared that erythrophores were strongly K^+ dose responsive based on *a** and *b** values. Lin, Oshio, Ohshima, Yananaka and Koizumi (1998b) previously reported that 480 mmol L^{-1} K^+ (but not 300 mmol L^{-1} K^+) caused a loss of red colouration from the skin of *P. major*. On the contrary, all concentrations used in this study affected red colouration; however, it appeared that erythrophores began to redisperse with storage time. Similarly, Lin, *et al*. (1998c) reported that erythrosomes in the red-coloured alfonsino *Beryx splendens* dispersed 10 h after death when soaked in seawater. While this study did not focus specifically on how K^+ initiated changes in chromatosome motility, it would appear that the presumed initial erythrosome aggregation caused by norepinephrine or K^+ depolarization of erythrophores was effective for several hours but it could not be sustained to that level for 48 h, in contrast with melanophores. Provided snapper are marketed at least 24 h after death, this return of pink colouration is favourable, as higher K^+ concentrations were required to obtain greater skin lightness.

While fish held in seawater ice slurries (0 mmol L^{-1} K^+) maintained the highest *a** and *b** values after death, an increase in chroma is generally associated with darker (i.e. lower *L** values) colouration (Doolan, *et al.* submitted [Chapter 4.5]). Therefore, to improve skin lightness by K^+ immersion, chroma is sacrificed to some extent to obtain light pink colouration rather than acquiring the vivid redness desired in *P. major* (Lin, *et al.* 1998b). Similar correlations in the colour characteristics of salmonid flesh have been displayed by Smith, Hardy and Torrissen, (1992). Therefore, in determining the optimal concentration of K^+ for post-harvest snapper skin colour improvement, 450 mmol L^{-1} K^+ is required to obtain significantly greater skin lightness than seawater slurries, and although reduced, a plateau in skin redness and yellowness was obtained from 300 mmol L^{-1}.

Although the results of this study recommend KCl addition for improvements in post-mortem skin colour, the Australia New Zealand Food Standards Code currently does not permit KCl as an additive in unprocessed fish or fish products. However, KCl is approved for use with processed fish and fish products, semi-preserved, and fully preserved fish including canned fish products. Despite its physiological function in changing the chromatic state of chromatophores rather than as a colourant from its own appearance, KCl used for the purpose in the present study is termed an additive under the code for its technological function in causing a colour change in the final product. Before KCl can be utilized in fish skin colour enhancement in Australia, approval must be obtained to using it as an additive. In countries where KCl is a permitted additive in unprocessed fish, its application may be transferable to other sparids that display dark colouration under sea cage conditions such as the red porgy *P. pagrus* (Rotllant, Tort, Montero, Pavlidis, Martinez, Wendelaar Bonga & Balm, 2003; Van der Salm, *et al.* 2004; Chatzifotis, *et al.* 2005) and *P. major* (Matsui, Tanabe, Furuichi, Yoshimatsu & Kitajima, 1992; Lin, *et al.* 1998a, b).

The results of this study suggest that K^+ should be supplied in seawater ice slurries at 300 – 450 mmol L^{-1} for cultured snapper when stored for 24 – 48 h on ice, but should not be used for short-term marketing Because of the initial loss of pinkness it promotes. In addition, K^+ supplementation in combination with conditioning dark-coloured snapper in white cages for 1 day before to harvest improves skin lightness considerably.

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TABLE 1
Summary of experimental phases

Phase	Description	N	Facilities	Duration	Water Quality
1. Pre-experiment	Snapper (761 ± 143 g; mean ± SD) held in black cages and fed 39 mg kg^{-1} unesterified astaxanthin	4 x replicate cages of 30 fish	4 x 1 m^3 black 9 mm knotless mesh cages suspended within a black 10000 L tank of recirculating system 2	150 days	Temperature 16.92 – 21.77 ^oC pH 7.54-8.33 Salinity 29.38 – 32.92 mg L^{-1} Dissolved oxygen 5.5-7.8 mg L^{-1}
2. Conditioning	Snapper (838 ± 141 g; mean ± SD) transferred to white or black cages for 1 day (W1, B1) or white cages for 7 days (W7)	5 x replicate cages of 1 fish for each of the 5 K^+ treatments (3 colour treatments x 5 K^+ treatments x 5 replicates = 75 cages)	50 x white and 25 x black cylindrical floating 0.2 m^3 cages suspended within 7 x 10000 L tanks of recirculating system 2	1, 7 days	Temperature 18.77 – 21.78 ^oC pH 8.17-8.34 Salinity 31.72 – 32.19 mg L^{-1} Dissolved oxygen 5.9-7.6 mg L^{-1}
3. K^+ experiment	Snapper from Phase 2 killed by ice slurry containing K^+ solutions of 0, 150, 300, 450 or 600 mmol L^{-1} K; fish removed after 1h, stored on ice and repeatedly measured	5 x replicate slurries of 1 fish for each of the 5 K^+ treatments (3 colour treatments x 5 K^+ treatments x 5 replicates = 75 slurries)	75 x 10 L buckets containing K^+ solutions	0, 3, 6, 12, 24, 48 h	

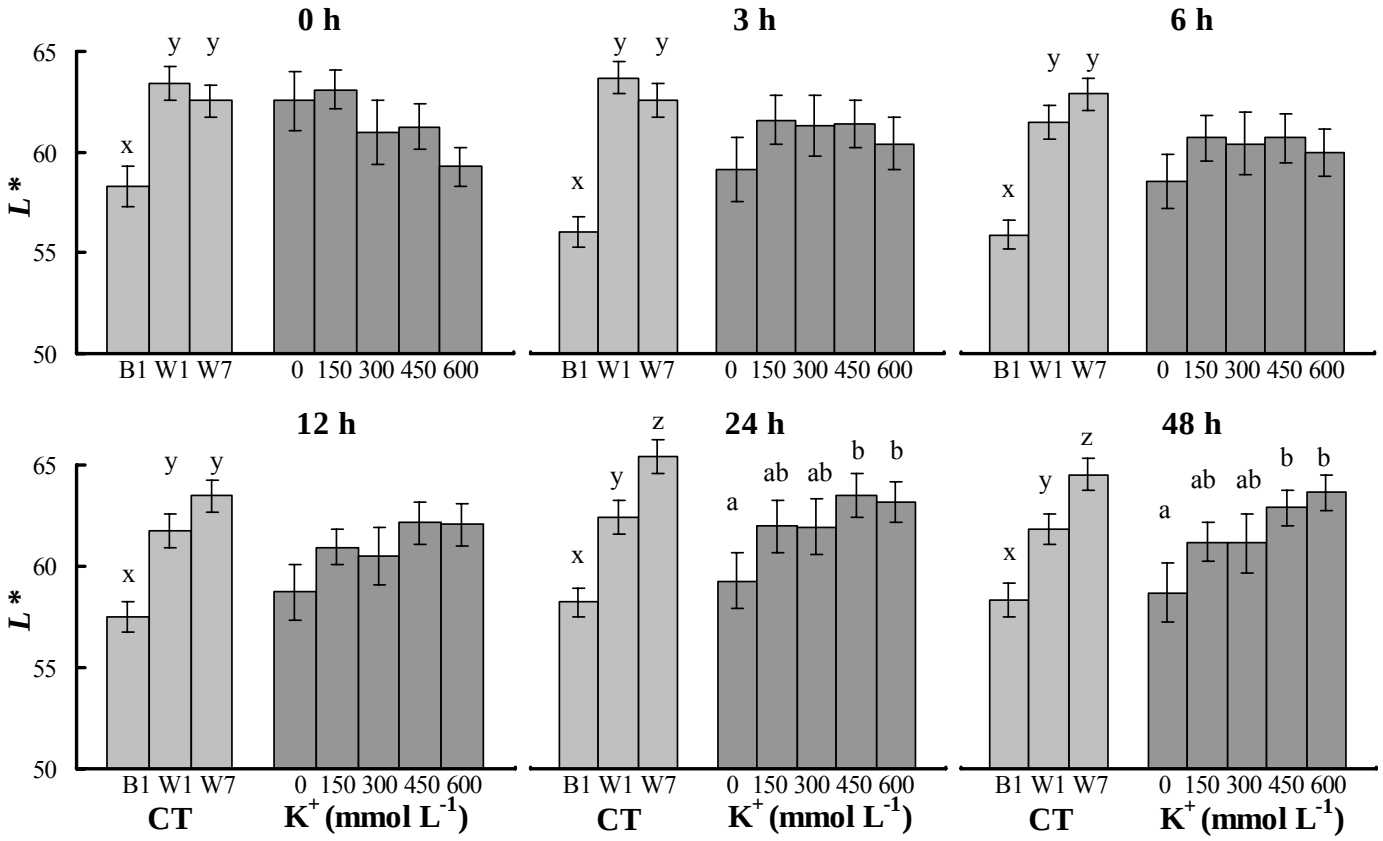

FIGURE 1
Summary of *L** values over time of snapper stored on ice following exposure to conditioning treatments (CT; B1, black 1 day; W1, white 1 day; W7, white 7 days) and K^+ concentrations. For each time post harvest, columns (level means ± SEM) within each factor (CT or K^+) with the same letter or where no letters are present denote no significant differences ($P > 0.05$, two-factor ANOVA, SNK).

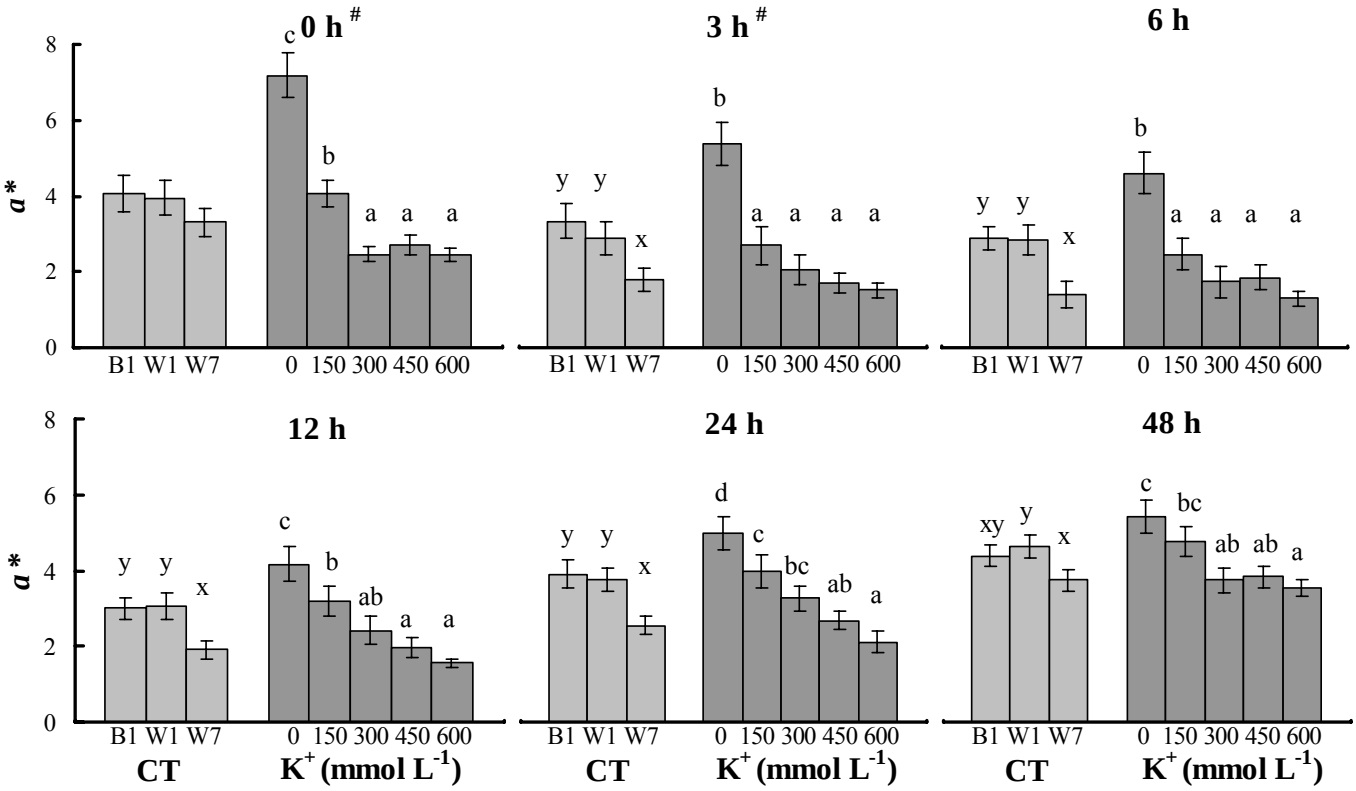

FIGURE 2
Summary of *a** values over time of snapper stored on ice following exposure to conditioning treatments (CT; B1, black 1 day; W1, white 1 day; W7, white 7 days) and K^+ concentrations. For each time post harvest, columns (level means ± SEM) within each factor (CT or K^+) with the same letter or where no letters are present denote no significant differences ($P > 0.05$, two-factor ANOVA, SNK). # Data arcsin transformed to homogenize variances and normalize data.

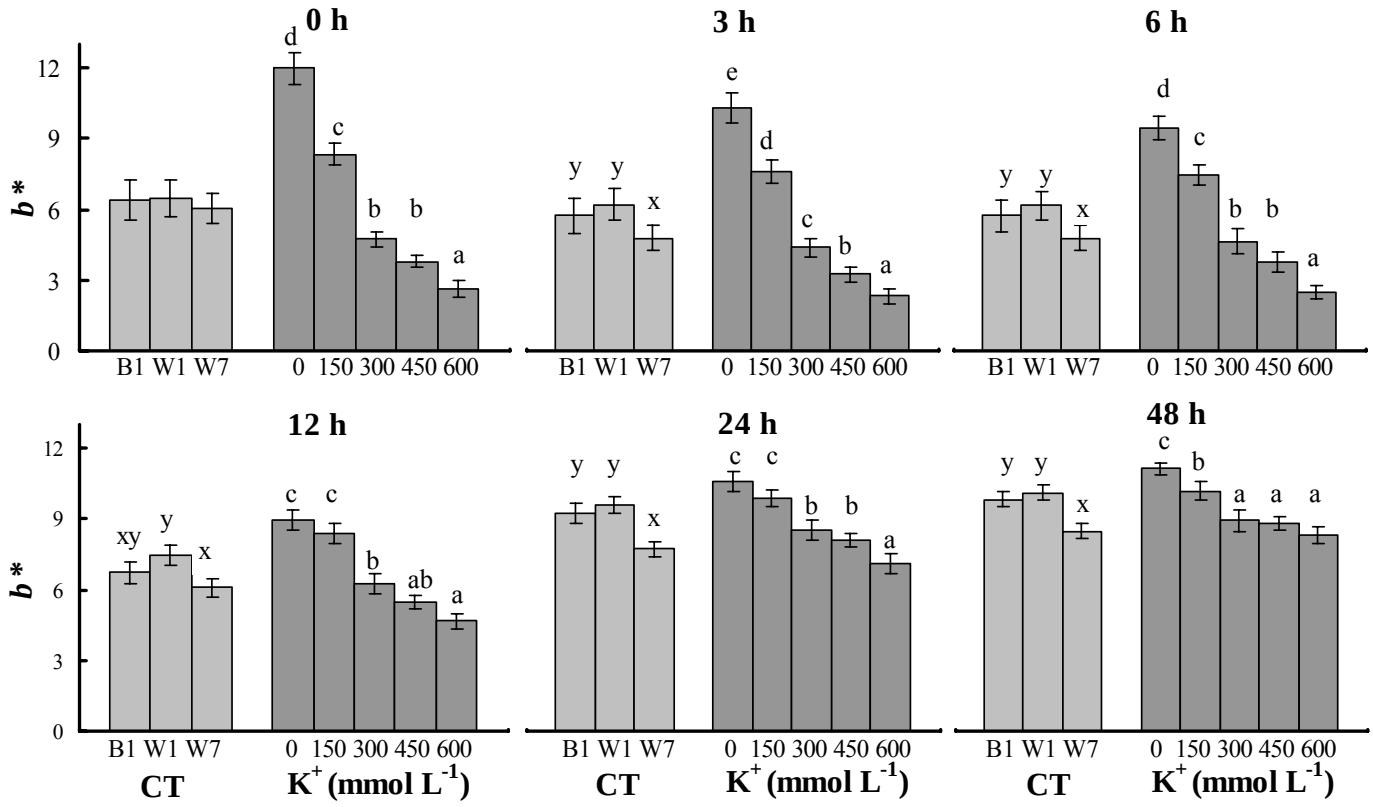

FIGURE 3
Summary of *b** values over time of snapper stored on ice following exposure to conditioning treatments (CT; B1, black 1 day; W1, white 1 day; W7, white 7 days) and K^+ concentrations. For each time post-harvest, columns (level means ± SEM) within each factor (CT or K^+) with the same letter or where no letters are present denote no significant differences ($P > 0.05$, two-factor ANOVA, SNK).

5. BENEFITS AND ADOPTION

- The research strategy of Aquafin CRC has been developed explicitly to deliver the essential technologies needed by the Australian finfish farming industry. The industry partners agreed to invest in a CRC, defined the major goals which they believed a CRC could best achieve, and clearly indicated the weight of effort which should be applied to each of these goals. These goals and weightings were first defined at a workshop of potential CRC participants in December 1999.

- The industry participants have continued to refine their priorities during the subsequent development and industry participants and researchers have met regularly (several times per year) to discuss results, implications of results and how they might be used, and refined plans for subsequent experiments.

- While there is currently no commercial snapper farming in Australia (fish farmers have moved to other faster growing marine fish like mulloway and yellowtail kingfish) the research has contributed in several major ways to Australian marine fish aquaculture:

    - Diets developed for snapper have helped form the basis for formulations for other marine carnivorous species.

    - One of the most significant benefits is the confidence among feed manufacturers to use alternative ingredients to fishmeal in response to reductions in availability and/or increases in price. The progression towards least-cost formulation (as is industry practice for terrestrial monogastric animal feed formulation) relies on rigorous ingredient evaluation, accurate estimation of nutritional requirements and systematic validation of different formulations.

    - Hatchery practices and nursery technology has been refined providing a much clearer understanding of the importance of abiotic factors, larval feeding strategies and diets on the cost-effective production of snapper fingerlings. Low-cost technology for extensive production of snapper larvae in fertilised ponds has also been developed. The new technology has been applied to other marine fish species providing an excellent starting point from which to refine larval rearing techniques for those species.

    - A clear understanding of how to improve skin colour of farmed snapper through manipulation of the culture environment and optimisation of dietary pigments.

    - Although a “solution” has not been found, the project has let to a much greater understanding of “velvet disease” caused by the parasitic dinoflagellate *Amyloodinium ocellatum* and best management practices to help avoid major problems.

    - The development of successful research methods cannot be underestimated as a benefit of this project. Research methods for diet development, larval rearing and nursery production and health management have all assisted in the design of new research to address similar problems for other species.

    - Research capacity at an institutional (NSW DPI and Ridley Aquafeeds) and personal level has been expanded.

- Adoption has been made possible because of the involvement of Ridley Aquafeeds throughout the project. The commercial fish farmers who were partners in the project are currently represented by Anthony O'Donohue (Clear Water Mulloway) who is presently using the Ridley Marine Fish Diet for production of mulloway and yellowtail kingfish.

6. FURTHER DEVELOPMENT

Despite the commercial investment in snapper farming at the commencement of this project, snapper farming has not developed in Australia. This is mainly because the economics of snapper culture, when the species was farmed as the only species in sea cage operations, were sub-optimal. Therefore it is of no surprise that, without exception, operators moved to faster growing species such as mulloway and yellowtail kingfish. However, even though snapper aquaculture has not developed, marine fish farming in Australia has expanded considerably. The research conducted during this project has assisted with the development of culture techniques for mulloway and yellowtail kingfish. In particular, results have been adapted during the Aquafin CRC project "Feed technology for temperate fish species" for both the hatchery/nursery and the diet development components, and information gained is being used by feed manufacturers and hatchery operators.

Assuming commercial production costs are equivalent for different diets, use of ingredients similar to those tested in this study can reduce the levels of fishmeal and thus the cost of snapper (and marine fish) feeds. Because of the high protein requirements of marine fish, the fact that productivity improvements can be achieved by feeding high-protein feeds and the increasing demand on existing fishmeal supplies, replacement of fishmeal in diets will be increasingly important in the future. During the course of this project, the cost of fishmeal has increased by at least 100% and at times has been virtually unavailable in Australia because of reductions in production and massive increases in demand, particularly from China. This rapidly changing supply/demand relationship for fishmeal has major implications for marine fish farming, particularly in Australia, where low production reduces the ability to negotiate large volume purchases of fishmeal at globally attractive prices. This reinforces the priority for continued investment in fishmeal replacement research.

In addition, because the "formulation space" for other energy sources will be reduced in nutrient dense feeds, the challenge is to identify and test high protein ingredients that in combination have a similar nutritional quality to fishmeal but at a lower cost. The paradox for Australian marine fish farmers is that rather than lowering the cost of feeds, high-protein nutrient dense feeds will inevitably cost more per kilogram than those formulated with a lower nutrient specification. However, the increased growth and improved FCR of this feeding strategy should make the use of more expensive, nutrient dense diets economically sound. The imperative for future research is to ensure whole-farm economics is considered when modelling different nutritional and feeding strategies. A key aspect is to ensure research is conducted with large fish most relevant to farming operations.

This project has led to considerable improvements in hatchery technology for snapper. Hatcheries dedicated to producing snapper are now able to produce nearly twice as many batches of snapper juveniles as they could using the "best practice" technology available at the start of the project. As the emphasis has shifted to other temperate marine fish species, e.g. mulloway, yellowtail and even southern bluefin tuna, it will be very important that hatchery procedures for each species are optimised. The research capacity developed during the project will be invaluable. In the past, meetings of marine fish hatchery managers and technicians have been very effective as a means of sharing new technology and identifying bottlenecks in production. Further hatchery development for marine finfish in Australia would benefit greatly from an expansion of this concept.

As production is intensified, health management will become increasingly important. The research during this project has progressed knowledge of velvet disease, caused by the parasitic dinoflagellate *Amyloodinium ocellatum.* However, other diseases will emerge to limit production. This is a critical area of research for future marine fish aquaculture development.

7. PLANNED OUTCOMES

- Profitable, expanding industry for snapper aquaculture in Australia. **Not met**. However, development of other marine fish farming industries has occurred and research methods and developed during this project, as well as research results, have assisted with the development of the marine fish culture industry in Australia.

- Viable hatcheries, breeding vigorous low-cost snapper fingerlings (for aquaculture or stock enhancement). Our goal is to reduce fingerling costs to around 25 cents/fish. (Specific outcome for Fingerling Production and Health Component). **Achieved.** Several marine fish hatcheries in Australia are now able to successfully produce snapper fingerlings and "commercial-scale" validation of new improved hatchery technology has occurred in South Australia, Western Australia and NSW.

- Development of techniques for management exclusion of ectoparasites in marine fish hatcheries. *Amyloodinium ocellatum* infests many fish species in hatcheries throughout the world. All Australian marine fish hatcheries will potentially benefit from this technology. (Specific outcome for Fingerling Production and Health Component). **Achieved.** While a successful treatment for *Amyloodinium ocellatum* was not developed, methods for excluding the problem from hatcheries and nurseries were developed and successfully applied.

- Commercially-available, cost-effective, high performance and low polluting diets for Australian snapper that help produce highly marketable fish of the desired colour. (Specific outcome for Diet Development and Skin Colour Component). **Achieved.** While there are no snapper being farmed at present, research results from the project have assisted the commercial feed manufacturer, Ridley Aquafeeds, with formulation of their Marine Fish Diet that is very effective for snapper. The Ridley Marine Fish Diet is recommended for grow out and the same diet with the addition of astaxanthin is recommended as a finisher diet to impart the desirable red pigment. A tank-based finishing treatment is recommended in addition to the pigmented finisher diet to ensure the best skin colour for farmed snapper.

- Increased availability of snapper for domestic (and export) markets. **Not achieved**. There has, however, been considerable expansion in production of other temperate marine finfish species and research and commercial experience with snapper has helped lay the foundation for this expansion.

8. CONCLUSIONS

- Dark-coloured Australian snapper *Pagrus auratus*, are less attractive to fish wholesalers and retailers and farmers can expect a discount of between 10 and 50% compared with light-coloured fish.
- Shading snapper from sunlight improved the lightness of their skin. Inclusion of astaxanthin in the diet as either the unesterified (Carophyll Pink™) or the esterified form (Naturose™) improved skin redness.
- The tank background colour has the major effect on snapper skin colour and the skin of dark-coloured snapper rapidly lightens after being exposed to white backgrounds.
- Reducing the intensity and altering the spectral profile of natural sunlight had minimal effect on skin lightness compared to the dominant effect of background colours.
- Snapper should be grown in white enclosures to maximise skin lightness. If this is not possible (e.g. because of sea cage culture), transfer of snapper to white enclosures for short periods before harvest for market is recommended.
- Of the commercially available carotenoids fed to snapper, astaxanthin (unesterified; Lucantin® Pink) was the best pigment, 39 mg unesterified astaxanthin kg^{-1} was the optimal dietary concentration and feeding astaxanthin diets for 42 days was the optimal time period for the best skin colour.
- Transfer of dark-coloured snapper to white backgrounds before live sale is highly beneficial and maximum change in skin lightness occurred within 1 day and did not elicit a stress response. If fish are to be harvested and stored for longer periods (e.g. 24-48 h) before sale, the skin of fish held in white backgrounds for only 1 day darkens; however, longer adaptation than 1 day can cause a small reduction in favourable red colouration.
- Skin colour of snapper grown in white net cages is much lighter than for fish grown in black net cages, but the beneficial effects are reduced through biofouling. Holding snapper at low stocking densities in backgrounds further improves skin lightness.
- Provided approval is obtained, K^+ supplied in seawater ice slurries at 300-450 mmol L^{-1} will reduce dark colouration of snapper skin, particularly when used in combination with holding fish in tanks with a white background before harvest. However, this is effective only when fish are to be stored for 24-48 h on ice and should not be used for short-term marketing Because of the initial loss of pinkness it promotes.
- Final recommendations are to feed snapper diets fortified with 39 mg unesterified astaxanthin kg^{-1} for 6 weeks to enhance pink pigmentation and then to hold fish before sale in white backgrounds for 1 day to maximise skin lightness.

9. APPENDICES

9.1 Intellectual Property

All information brought into this project or developed during the project is public domain.

9.2 Staff

- Dr Geoff L. Allan, Principal Investigator/Research Leader, Aquaculture and Port Stephens Fisheries Centre Director.
- Dr Mark A. Booth, Co-Investigator/Research Scientist, Fish Nutrition, Port Stephens Fisheries Centre.
- Dr Stewart Fielder, Co-Investigator/Research Scientist, Marine Fish Breeding, Port Stephens Fisheries Centre.
- Mr Ben J. Doolan, Fisheries Technician, Port Stephens Fisheries Centre; PhD student, Deaking University
- Mr Luke Cheviot, Fish Hatchery Manager, Port Stephens Fisheries Centre.
- Mr Ian Russell, Fisheries Technician, Port Stephens Fisheries Centre.
- Mr Peter Dickson, Fisheries Technician, Port Stephens Fisheries Centre.
- Ms Rebecca Warner-Smith, Fisheries Technician, Port Stephens Fisheries Centre.
- Mr Bill Bardsley, Fisheries Technician, Port Stephens Fisheries Centre.
- Ms Geraldin McMahon, Fisheries Technician, Port Stephens Fisheries Centre.
- Ms Debra Ballagh, PhD Student, James Cook University.
- Dr Paul Jones, Deakin University.