Development of vision and larval feeding responses in southern bluefin tuna and yellowtail kingfish

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Abstract

Southern bluefin tuna, *Thunnus maccoyii* and yellowtail kingfish, *Seriola lalandi*, are marine finfish species currently cultured in Australia. High early mortality and larval malformations have hindered the successful production of quality juveniles. Rearing conditions experienced during early larval culture are critical for the production of high quality seed stock. My study described the visual capacity of *T. maccoyii* and *S. lalandi* to feed under a variety of abiotic and biotic conditions and examined retinal morphology and physiology to identify species-specific adaptations to help explain the observed feeding behaviour. The morphological development of the visual apparatus of *T. maccoyii* and *S. lalandi* is described by histological analysis and microspectrophotometry (MSP) and the visual ability of larvae is examined through behavioural experimentation. Larvae were visually challenged to feed under a range of conditions in short-duration (4 h) first-feeding experiments. Feeding performance was measured as the proportion of larvae feeding and the intensity of feeding. In *T. maccoyii* the first-feeding performance was positively affected by increasing prey density and lower turbidities and unaffected by light intensity, tank colour, turbulence, prey size and larval density. In contrast, *S. lalandi* showed greater limitation in terms of feeding performance, and in the number of variables that were conducive to feeding, which indicated a narrower “environmental window” for first-feeding success. Light intensity, tank colour, turbulence, larval density and prey density all significantly affected the feeding response in early-feeding *S. lalandi*. Feeding experiments on 3, 6 and 9 dph larvae revealed that as *T. maccoyii* aged, lower light intensities significantly increased feeding performance, indicating increased photopic sensitivity. In contrast, *S. lalandi* continued to display better feeding performance with increasing light intensity and age. Histological studies revealed significant differences between the retinal indices of *T. maccoyii* and *S. lalandi* larvae. *Thunnus maccoyii* displayed high cell densities in the ventral region of the eye, possessed a low convergence of cone cells onto ganglion cells, with relatively large cones at first feeding (almost twice the size of *S. lalandi*

first-feeding larvae) and exhibited retinal pigment epithelium (RPE) pigment migration at an early age (12 dph). *Seriola lalandi* displayed high cell densities in the dorsal retinal region and on average possessed twice the convergence of cones onto ganglion cells. Retinal pigment epithelium migration in *S. lalandi* was not seen until the development of rods at 21 dph. MSP and behavioral feeding experiments under coloured lights showed that *T. maccoyii* displayed peak spectral sensitivity in the blue spectrum, whereas *S. lalandi* displayed sensitivity in the red and green spectrum. My study has also identified important differences between the species that have culture implications. The strong first-feeding response of *T. maccoyii* across a broad range of abiotic and biotic factors indicates that major mortality during their early life history is not associated with a failure to initiate feeding. Although, *T. maccoyii* are likely to show decreased feeding and increased surface and sinking mortality, when the larvae are exposed to light intensities commonly encountered in the culture of fish larvae. The use of low-light intensity during larval rearing has the potential to dramatically improve larval survival during the first two weeks of culture. In comparison, while *S. lalandi* displayed improved feeding with increasing age, the narrow set of parameters that were conducive to first-feeding highlighted the need for strict control of culture parameters during this critical stage.

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Dedicated to my beautiful angels Ella and Maya Hilder

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Chapter 1. General introduction

1.1 Background

Global demand of fish for human consumption is outstripping wild supply to the extent that 32% of the world's fish stocks are now suffering negative effects from over-fishing, and 50% of the remaining stocks are fully exploited (FAO, 2010). To meet the increasing consumer demand, which cannot be met by the exploitation of wild fish stocks, there has been heavy investment into the development of aquaculture industries. In 2009, aquaculture production reached 55 million tonnes and production is predicted to outstrip wild supply in the future (FAO, 2010).

Southern bluefin tuna, *Thunnus maccoyii*, in Australia, and yellowtail, *Seriola quinqueradiata*, in Japan, are both economically important aquaculture industries based on the capture of wild juveniles that are fattened for market (Nakada, 2002; Thomson et al., 2010) In 2004, the Japanese *Seriola* sp. industry was valued at US $1.2 billion (Nakabo, 1993; Nakada, 2000) and in 2009, the *T. maccoyii* industry in Australia was valued at AUD $157.8 million (ABARE, 2010). The culture technology for on-growing of *T. maccoyii* and *S. quinqueradiata* is well established and both species domesticate well, have rapid growth rates, excellent flesh quality and a high market demand, highlighting the suitability of these species for aquaculture production (Benetti et al., 2005; Kolkovski and Sakakura, 2004; Thomson et al., 2010).

The reliance on wild caught juveniles for aquaculture production, however, has a number of limitations when compared to closed life cycle rearing in captivity. The culture of domestically spawned embryos allows stock improvement through selective breeding and extension of the production season through reproductive manipulation. Economic viability is directly increased through higher production outputs, increased hatchery utilisation and faster response time to market demands.

In Australia and New Zealand, intense scientific research has been invested into the control of reproduction in *T. maccoyii* and yellowtail kingfish, *Seriola lalandi* (Elizur et al., 2009; Gillanders et al., 1999; Moran et al., 2007b; Poortenaar et al., 2001; Thomson et al., 2010). Adult *T.*

maccoyii and *S. lalandi* broodstock have responded well in captivity and produced viable embryos over a number of spawning seasons (Cobcroft, 2013; Cobcroft et al., 2012b).

Development of a *T. maccoyii* aquaculture industry in Australia, from spawned embryos, has been constrained by a bottleneck in the production of larvae beyond 14 days post-hatching (dph) (Cobcroft et al., 2012b; Hutchinson, 2009). In contrast, *S. lalandi* juveniles are cultured in large numbers, with production expected to reach 5000 tonnes per annum by 2020. Even with this success, however, major problems with malformation and swimbladder inflation are thought to be costing the industry AUD $1 million per annum, constraining the development of the industry (Battaglene and Cobcroft, 2007; Cobcroft, 2013).

The major bottlenecks experienced in the culture of *T. maccoyii*, and *S. lalandi* are problems that are not only faced in Australia. While the life cycle of the closely related Pacific bluefin tuna, *Thunnus orientalis*, in Japan is closed and the industry is capable of producing tens of thousands of ex-hatchery juveniles, mortality is still a major problem during larviculture (Masuma et al., 2011). Cultured larval and juvenile *Seriola* sp. in Japan and New Zealand, also exhibit high deformity rates and low swim bladder inflation (Benetti et al., 2005; Kolkovski and Sakakura, 2004; Woolley et al., 2012a).

Evidence from larval rearing trials in Australia would indicate that the causative factors leading to high mortality in *T. maccoyii*, and malformation and lack of swimbladder inflation in *S. lalandi*, occur during the first two weeks of culture (Cobcroft, 2013; Cobcroft et al., 2012b). A greater understanding of *T. maccoyii* and *S. lalandi* early larval development and definition of optimum early rearing parameters are required in order to solve the major bottlenecks in quality seed production.

1.2 Why a comparative study?

While differences between *T. maccoyii* and *S. lalandi* become evident with increasing size, in particular the explosive growth observed in post-larval

T. maccoyii, they share a very similar early life history (Cobcroft et al., 2012b; Fielder et al., 2010; Fujioka et al., 2010). Both are progeny of pelagic broadcast spawners that hatch in an early stage of ontogeny reliant on endogenous energy reserves until 3 dph when first-feeding commences (Cobcroft et al., 2012b; Fielder et al., 2010; Fujioka et al., 2010). The species also share similar larval growth and morphological development during the first week post-hatching (Hutchinson, 2009; Woolley and Qin, 2013; Woolley et al., 2013). *Thunnus maccoyii* and *S. lalandi* both develop rapidly and reach the juvenile stage with the development of sensory, digestive, structural systems (including ossification of bone) and fins and musculature being completed within 30 dph (Battaglene and Cobcroft, 2007; Chen et al., 2007; Cobcroft and Pankhurst, 2006; Cobcroft et al., 2012a; Houde and Schekter, 1980; Woolley and Qin, 2013; Woolley et al., 2013). While the larvae undergo this rapid development, the physiological and structural changes that the larvae experience are affected by the aquatic environment, particularly during the early stages of development (Battaglene and Cobcroft, 2007; Cobcroft et al., 2012b; Hutchinson, 2009). The behavioural responses and mortality of *T. maccoyii* and *S. lalandi* displayed in culture do not reflect similar patterns, indicating a difference in culture requirements. In general, a species' physiological and morphological make-up reflects the environment in which they live, thereby increasing the odds of survival in that given environment (Guthrie and Muntz, 1993). As larvae display species-specific needs, exposure to sub-optimal conditions can result in detrimental physiological and/or behavioural outcomes that, in culture, lead to malformation, poor growth, lack of swimbladder inflation, disease and mortality (Bristow et al., 1996; Chen et al., 2007; Cobcroft et al., 2012b; Downing and Litvak, 2001; Woolley et al., 2012a). Optimal rearing conditions experienced during larval culture are critical for the production of high quality seed stock. The different behavioural responses and mortality of the larvae observed in *T. maccoyii* and *S. lalandi* culture, highlight the need for a greater understanding of larval requirements in order to improve larviculture protocols and the subsequent success of their respective aquaculture industries (Battaglene and Cobcroft, 2007;

Cobcroft, 2013; Cobcroft et al., 2012b; Woolley et al., 2013). As vision is the primary sense for feeding in marine fish larvae (Blaxter, 1986), understanding visual ontogeny and factors that affect the visual environment are critical in understanding larval requirements and furthering the larviculture of both species.

1.3 Objectives

The overall aim of my study was to compare and contrast the visual development and visual capacity of *T. maccoyii* and *S. lalandi* during larval ontogeny. This information can then be used to explain and inform the best rearing conditions to produce high quality seed stock. The specific aims of the study were:

- To determine the effect that the visual environment has on the first-feeding of *T. maccoyii* and *S. lalandi* larvae, by testing the effect of light intensity, turbidity, turbulence, tank colour, prey density, larval density and prey size on the feeding response.
- To determine how light intensity and prey density affect the feeding response of *T. maccoyii* and *S. lalandi* as the visual system of the larvae develops.
- To compare the visual ontogeny of *T. maccoyii* and *S. lalandi* in order to identify the developmental sequences and the species-specific retinal adaptations.

To achieve my aims I undertook a multidisciplinary experimental approach. This included histological examination of retinal anatomy in order to map retinal ontogeny and identify species-specific retinal adaptations, microspectrophotometry of retinal photoreceptors to identify spectral sensitivity, and behavioural analysis of feeding performance over a broad range of environmental variables to identify conditions that are conducive to early feeding. To my knowledge, this is the first study to investigate *Thunnus* sp. and *Seriola* sp. using a comparative approach.

1.4 Study site

Thunnus maccoyii and *S. lalandi* larval rearing and behavioural feeding experiments were conducted at Clean Seas Tuna Ltd, Arno Bay, South Australia (Fig. 1) between 2010 and 2012. Preserved samples for histological analysis were processed at the Institute for Marine and Antarctic Studies (IMAS), University of Tasmania, with microspectrophotometry being completed at the University of Western Australia.

Figure 1. Map of Australia showing the location of Clean Seas Tuna Ltd, Arno Bay, South Australia, IMAS, Tasmania and the University of Western Australia.

1.5 Species description

1.5.1 Thunnus maccoyii

Thunnus maccoyii (Fig. 2) are large (up to 200 cm and 200 kg) migratory fish with a circum-global distribution in the southern regions of the western -Atlantic, western-Pacific and Indian oceans (Caton, 1994; Davis et al., 1991). Mature fish are known to travel in excess of 8000 km during migration to their spawning grounds (Davis and Farley, 2001; Patterson et al., 2008) in the northeastern Indian Ocean south of Java (Fig.3), between September and April (Itoh and Tsuji, 1996). There is limited information available regarding larval *T. maccoyii* in the wild, as larvae are only

observed irregularly, in low density and with patchy distribution (Davis et al., 1991). It is thought that larvae complete their larval cycle in the spawning grounds in warm waters where initial growth is relatively slow and similar to temperate species (Jenkins and Davis, 1990).

Figure 2. A large adult southern bluefin tuna, *Thunnus maccoyii* (photo courtesy of Adam Miller, Clean Seas Tuna Ltd).

The consumption of prey by *T. maccoyii* larvae in the wild has been positively correlated with mouth gape, thereby displaying improved feeding energetics with increasing larval size, with the main prey items being copepod nauplii, calanoids, cyclopoids and cladocerans (Young and Davis, 1990). The growth rate of larval *T. maccoyii* is positively correlated with feeding rate but not temperature (Jenkins et al., 1991). Rapid growth in young tuna does not occur during the early larval stages and is thought to occur during the late larval and early juvenile stages (Jenkins and Davis, 1990). Juvenile fish depart the spawning grounds and commence a southerly migration along the western and southern coast of Australia prior to dispersal throughout the southern oceans as mature fish (Jenkins et al., 1991).

Thunnus maccoyii can live to 40 years of age and do not reach sexual maturity until their tenth year, which leaves them particularly vulnerable to

over-fishing (Caton, 1994; CCSBT, 2011; Patterson et al., 2008). Since commercial fishing for *T. maccoyii* commenced in the 1950's (Caton, 1994) the population has been reduced by 92%, with current spawning biomass at a fraction of its original biomass (CCSBT, 2011). This has resulted in the placement of *T. maccoyii* on the critically endangered list by the International Union for the Conservation of Nature (IUCN, 2012) and the *T. maccoyii* fishery being subject to management quotas. The introduction of the quota system has resulted in a reduction in the total reported global catch of *T. maccoyii* and it is expected that stocks will begin to increase if current catch levels are maintained (CCSBT, 2011).

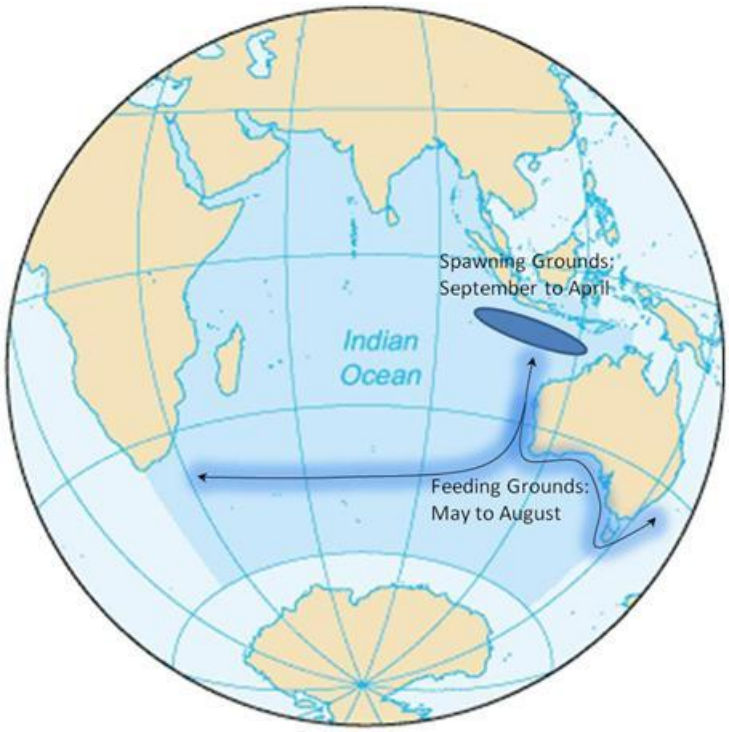
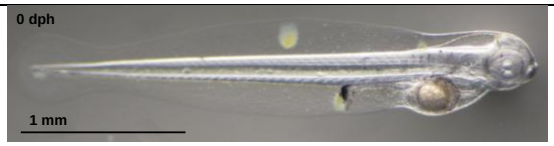
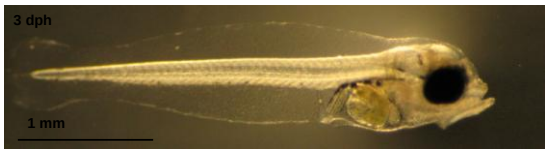
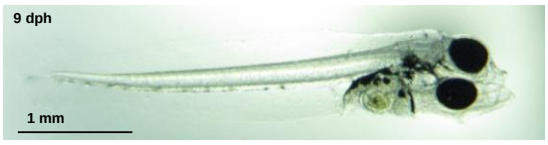
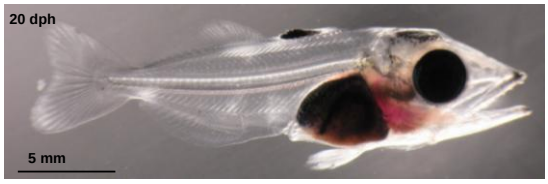


Figure 3. Distribution map of southern bluefin tuna, *Thunnus maccoyii*, indicating spawning and feeding grounds (Illustration provide by N. Kirchhoff, Australian Maritime College).

Aquaculture of *T. maccoyii* in Australia was established in 1991 (CRC, 2013) based on the capture of wild juveniles for on-growing and fattening for market. The culture of fattened *T. maccoyii* accounts for 12% of all Australian aquaculture production (ABARE, 2010). As the Australian *T. maccoyii* industry is restricted by total allowable catch quotas, expansion of the industry through the culture of *T. maccoyii* from embryos is being

investigated (Thomson et al., 2010). *Thunnus maccoyii* were successfully spawned in captivity in 2008 with subsequent larviculture trials commencing in 2009 (Hutchinson, 2009; Thomson et al., 2010). To date, the development of a *T. maccoyii* aquaculture industry based on culture from embryo has been unsuccessful, predominantly due to the high larval mortality (Cobcroft et al., 2012b; Hutchinson, 2009). Mortality is often observed as surface mortality or sinking mortality during the first two weeks of culture and generally 95% of the cohort is lost by 20 dph (Table 1) (Hutchinson, 2009).

Table 1. *Thunnus maccoyii* developmental stage and associated mortality.

Larval age	Development stage	Mortality (days post-hatching)
	Hatch	Surface (0 – 3)
	First-feeding swimbladder inflation	Surface (3 – 6)
	Early exogenous feeding	Sinking (3 – 12)
	Post - flexion	Cannibalism (>15)
	Juvenile	Collision (> 30)

Photos by P. Hilder. Information obtained from Hutchinson, 2009 and Cobcroft, et al., 2012b.

There is little documented information on the culture requirements of *T. maccoyii*, although intense scientific interest has concentrated on closely related tuna species, *T. orientalis*, yellowfin tuna, *Thunnus albacores,* and the northern bluefin tuna, *Thunnus thynnus* (Table 2).

Table 2. Literature investigating the culture of larval *Thunnus maccoyii, Thunnus orientalis, Thunnus albacores and Thunnus thynnus* (sorted by genus).

Author	Species	Study
Cobcroft, et al., 2012b	*T. maccoyii*	Larval rearing
Fielder et al., 2009	*T. maccoyii*	Embryo disinfection
Hutchinson, 2009	*T. maccoyii*	Larval rearing advances
Thomson, et al., 2010	*T. maccoyii*	Larval rearing
Woolley et al., 2009	*T. maccoyii*	Larval hatching
Woolley et al., 2013	*T. maccoyii*	Swim bladder
Fukuda et al., 2010	*T. orientalis*	Retinomotor response
Ishibashi et al., 2009	*T. orientalis*	Night-time mortality
Ishibashi, 2010	*T. orientalis*	Seedling production
Kaji et al., 1996	*T. orientalis*	Digestive development
Kato et al., 2008	*T. orientalis*	Effect of turbulence
Kawamura et al., 2003	*T. orientalis*	Development of vision
Kurata et al., 2012	*T. orientalis*	Swimbladder inflation
Masuma et al., 2001	*T. orientalis*	Retinomotor response
Masuma et al., 2011	*T. orientalis*	Review larviculture
Matsuura et al., 2010	*T. orientalis*	Visual cell development
Miyashita et al., 2001	*T. orientalis*	Morphological development
Miyashita, 2002	*T. orientalis*	Seed production
Nakagawa et al., 2011	*T. orientalis*	Aeration and survival
Nakamura et al., 2013	*T. orientalis*	Visual pigments
Sabate et al., 2010	*T. orientalis*	Schooling and cannibalism
Sakamoto and Nakagawa, 2009	*T. orientalis*	Turbulence control
Sawada et al., 2005	*T. orientalis*	Closing the life cycle
Tanaka et al., 2009	*T. orientalis*	Sinking mortality
Tanaka et al., 2012	*T. orientalis*	Prey utilisation
Torisawa et al., 2007	*T. orientalis*	Retinomotor response
Torisawa et al., 2011	*T. orientalis*	Schooling behaviour
Kimura et al., 2004	*T. albacares*	Effect of turbulence
Loew et al., 2002	*T. albacares*	Visual pigments
Margulies, 1997	*T. albacares*	Visual development
Margulies et al., 2007a	*T. albacares*	Review early life history
Margulies et al., 2007b	*T. albacares*	Early development
Partridge et al., 2011	*T. albacares*	24 h photoperiod
Wexler et al., 2011	*T. albacares*	Temperature and oxygen
Zink et al., 2011	*T. albacares*	Larval transport
Caggiano et al., 2009	*T. thynnus*	Larval rearing
Masuma et al., 2011	*T. thynnus*	Broodstock and larvae
Miyashita et al., 2000	*T. thynnus*	Collision mortality

1.5.2 Seriola lalandi

Seriola lalandi (Fig. 4) is a circum-global species occurring in tropical-temperate waters of the northern and southern hemisphere, but not around the equator (Kolkovski and Sakakura, 2004; Smith, 1987).

Figure 4. Yellowtail kingfish, *Seriola lalandi* (photo courtesy of Adam Miller, Clean Seas Tuna Ltd.).

Fish can reach a total length of 150 cm and weigh up to 50 kg (Benetti et al., 2005). Sexual maturity is attained between 75 – 92.5 cm in males and 77.5 – 127.5 cm in females (Poortenaar et al., 2001). Adult fish inhabit coastal areas associated with rocky reefs and islands, with repeated spawning occurring during the spring and summer months (Gillanders et al., 1999; Moran et al., 2007b; Smith, 1987). Larval *S. lalandi* are generally associated with coastal waters, however, they can also be found up to 320 km offshore, and juveniles (< 30 cm) are commonly found near or beyond the continental shelf aggregated beneath floating debris (Kolkovski and Sakakura, 2004; Smith, 1987; Sumida et al., 1985).

Seriola sp. is a global aquaculture genus cultured in Asia, South America and Australasia (Nakada, 2002; Poortenaar et al., 2001). In Japan, the culture of *S. quinqueradiata* accounts for over 90% of the total aquaculture industry, although this is primarily based on the on-growing of wild caught juveniles (Nakabo, 1993; Nakada, 2008). In contrast, aquaculture production of *S. lalandi* in Australia and New Zealand is based on the rearing of embryos produced from domestic broodstock (Kolkovski and Sakakura, 2004; Poortenaar et al., 2001). *Seriola lalandi* culture

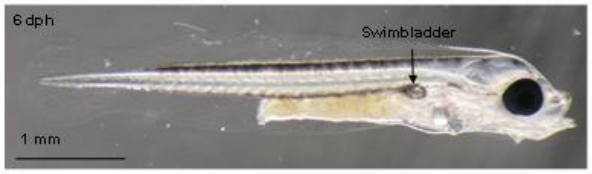
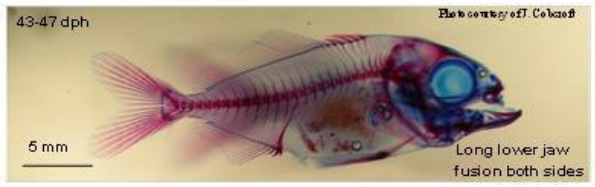
commenced in Australia in the 1990's with early success in the control of regular spawning events resulting in the establishment of *S. lalandi* hatcheries in Western Australia, South Australia and New South Wales (Benetti et al., 2005). A number of studies have investigated larval development and culture (Table 3).

Table 3. Selected studies investigating the culture of larval *Seriola lalandi*.

Author	Study
Battaglene and Cobcroft, 2007	Juvenile quality
Benetti, et al., 2005	Culture review
Carton, 2005	Light intensity and turbidity on larvae
Carton and Vaughan, 2010	Visual acuity
Chen et al., 2006b	Digestive development
Chen et al., 2006a	Digestive development
Chen, et al., 2007	Deleterious food restrictions
Cobcroft, et al., 2004	Jaw malformation
Cobcroft, 2013	Improved larval production
Hilton et al., 2008	Embryogenesis
Kolkovski and Sakakura, 2004	Culture review
Ma and Qin, 2012	Rotifer enrichment for larval rearing
Miyagi et al., 2001	Visual acuity in 15 mm to 390 mm fish
Moran et al., 2007a	Energetics and metabolism of eggs
Moran et al., 2007b	Reproduction and early development
Nakada, 2002	Culture review
Poortenaar et al., 2000	Kingfish culture in NZ
Stuart et al., 2010	Egg disinfection
Stuart and Drawbridge, 2011	Light intensity and turbidity on larval fish
Stuart and Drawbridge, 2012a	Larval culture
Stuart and Drawbridge, 2012b	Photoperiod
Woolley et al., 2012a	Swimbladder inflation and growth
Woolley et al., 2012b	*Artemia* sp. feeding and mortality
Woolley and Qin, 2013	Body density

While *S. lalandi* larvae can be produced in relatively high numbers, poor swimbladder inflation and high deformity rates significantly affect quality seed production restricting the commercial success of this species (Table 4) (Kolkovski and Sakakura, 2004; Woolley et al., 2012a).

Table 4. *Seriola lalandi* developmental stage and associated culture problems.

Larval age	Development stage	Complication (days post-hatching)
	Hatch	Hatching mortality (0)
	First-feeding	First-feeding mortality (> 3)
	Swimbladder inflation	No inflation (> 3)
	Juvenile	Jaw and spinal deformity (> 10)

Photos by P. Hilder. Information supplied from Battaglene and Cobcroft, 2007 and Cobcroft, 2013.

1.6 The sensory organs

The sensory organs in fish include optical receptors, mechanoreceptors, audioreceptors and chemoreceptors (Kjørsvik et al., 2004) The receptors allow larval fish to identify prey and predators from the surrounding environment through the detection of physical and chemical cues (Blaxter, 1986). Sensory development in larval fish is progressive, with different rates of development observed in larvae with direct, indirect or intermediate development (Kjørsvik et al., 2004). Regardless of the rate of sensory development, larvae become equipped with the necessary sensory function for the detection of prey and predators. Sensory morphology often correlates to the habitat of the fish and the timing of sensory development has been suggested to coincide with species-specific habitat shifts e.g., metamorphosis when some species make the transition from a pelagic to benthic lifestyle (Batty and Hoyt, 1995; Higgs

and Fuiman, 1998; Kawamura et al., 2003). Fish may be categorised as either sensory specialists (reliant on a predominant sense), or sensory generalists (larval response is dependent upon the available sense) (Higgs and Fuiman, 1998; Poling and Fuiman, 1998). While sensory specialists do rely on a predominant sense e.g., vision, they often use a combination of the senses for feeding which may include the visual detection of prey (optical reception), olfaction of prey (distance chemoreception), movement of prey (mechanoreception), sound (audioreception) and gustation (direct chemoreception) (Batty and Hoyt, 1995). For many fish, particularly pelagic species, vision is the predominant sense for feeding (Blaxter, 1986). For species that feed at depth or at night, in the absence of light, feeding is reliant on the non-visual senses (Jones and Janssen, 1992). For example, sole, *Solea solea*, a nocturnal species, is totally reliant on chemoreception and mechanoreception for feeding, whereas larval red drum, *Sciaenops ocellatus*, which lives in a number of microhabitats, display equal responsiveness regardless of which sensory system is available (Batty and Hoyt, 1995; Poling and Fuiman, 1999).

1.7 Development of sensory capability

1.7.1 Optical receptors

Marine fish larvae generally hatch in an undeveloped state with non-functional eyes incapable of image formation (Blaxter, 1975). In order for first-feeding larvae to make the transition from endogenous to exogenous feeding, rapid morphological development of the visual system is required (Blaxter, 1986). The first-feeding larva possesses a small eye which constrains the amount and type of retinal tissue present (Kotrschal et al., 1990). The small eye generally contains a single type of photoreceptor i.e., cones (a simplex retina). This restricts feeding to conditions of relatively high light intensity but does provide photopic acuity (Evans and Fernald, 1990; Kotrschal et al., 1990; Sandy and Blaxter, 1980). Visual acuity is the amount of detail that can be resolved by the eye (Fernald, 1989; Fritsches et al., 2003; Neave, 1984), and is of primary importance to

larval fish in order to detect and capture live prey, as failure to do so results in mortality due to starvation (Hjort, 1914; May, 1974). While larvae possess photopic acuity at first-feeding, the ability to forage under low light intensities (scotopic sensitivity) is restricted as the small eye cannot accommodate parallel development of photopic and scotopic visual apparatus (Blaxter, 1986; Kotrschal, et al., 1990). As the larval eye increases in size, stretching of the retinal tissue provides more room for retinal development (Johns, 1981). Generally, visual ontogeny commences with the enlargement of single cones increasing photon capture and visual acuity (Vandermeer, 1994). The recruitment of new photoreceptor classes increases the sensitivity of the eye as the addition of double cones provides improved resolution at lower light levels and the development of rods (duplex retina) is associated with movement perception and scotopic vision (Blaxter, 1986; Blaxter and Staines, 1970; Evans and Fernald, 1990; Pankhurst and Hilder, 1998). The rearrangement of cones to form cone mosaics results in increased colour resolution, contrast, visual acuity and improved motion perception, which is of primary importance in predatory fish (Ahlbert, 1973; Fernald, 1989). The development of the retinomotor response, generally coinciding with the development of a duplex retina, allows control of the amount of light that reaches the retina (Blaxter and Jones, 1967; Jobling, 1995). As ontogeny proceeds the visual capacity improves, consequently much of the larval life is spent with a visual system inferior to that of the adult (Kotrschal, et al., 1990).

The timing of the overall sequence of photoreceptor development is species-specific and correlates to intra-specific physiological and behavioural changes (Branchek and Bremiller, 1984). For this reason it is critical to gain a thorough knowledge of retinal development in order to understand factors that affect visually mediated behaviour, such as feeding success, throughout larval development.

1.7.2 Mechanoreceptors

Newly hatched marine fish larvae generally have free neuromasts prevalent in the head region that increase in number with ontogeny and develop along the side of the larvae prior to the encasement in the lateral line at metamorphosis (Kawamura et al., 2003; Kjørsvik et al., 2004). Improved motion detection has been identified in larval fish which possess a higher number of free neuromasts and larger cupulae, and in general, pelagic fish larvae exhibit a greater number of free neuromasts than demersal fish larvae (Kawamura et al., 2003). Well-developed mechanoreceptors have been reported in the pelagic larva *T. orientalis*, which would provide increased motion detection (Kawamura et al., 2003). In culture, the highly developed mechanoreception may cause undue stress with excessive handling, vibrations, aeration and turbulence Kawamura et al., 2003).

1.7.3 Audioreceptors

The inner ear facilitates the detection of vibration by sensitivity to acoustic stimuli, while also providing the fish with equilibrium (Kawamura et al., 2003; Lagler et al., 1962). At hatch, the otoliths are present in the inner ear of larvae (Lagler et al., 1962). However, it is not until the development of cilia on the hair cells, and the subsequent detection of differential movement between the cilia and otoliths, that the inner ear is thought to be functional (Kawamura and Ishida, 1985; Lagler et al., 1962). The timing of inner ear functionality varies between species, and can be present at hatching as reported in *T. orientalis*, or develop during the larva's early life history generally prior to first feeding as seen in the striped trumpeter, *Latris lineata* and the Japanese flounder, *Paralichthys olivaceus* (Cobcroft and Pankhurst, 2003; Kawamura and Ishida, 1985; Kawamura et al., 2003*)*. The degree of auditory function is thought to be influenced by the number and density of hair cells, as seen in the zebrafish, *Danio rerio*, which display a direct correlation between increased auditory function and hair cell number during the first week of development post hatching (Lu and DeSmidt, 2013). Larvae with swimbladders in close proximity or

mechanically connected to the inner ear have also been shown to have an increase in acoustic ability (Lagler et al., 1962). The swimbladder changes in volume in response to sound pressure waves and it is the movement of the swimbladder wall that is transmitted to the inner ear via proximity, or by the Weberian ossicles (Kawamura et al., 2003; Lagler et al., 1962).

1.7.4 Chemoreceptors

Chemoreception in fish is provided through the olfactory and taste systems. The development of the olfactory organ is reviewed in Kasumyan (2011) and is described as the proliferation of olfactory receptor cells located at the base of the olfactory pits to form the primordium of the olfactory organ. As this organ increases in size, folds arise and eventually form the olfactory rosettes. The olfactory organs, or nares, are initially flat structures and with development the organ descends and skin flaps rise laterally and fuse together to form a channel above the organ, allowing detection of chemostimuli from the direction water enters the channel (Kjørsvik et al., 2004; Pankhurst and Butler, 1996).

Olfactory detection of zooplankton by fish larvae is thought to drive chemotactic behaviour, due to the release of metabolites by the zooplankton that are high in amino acids (Corner and Davies, 1971; Døving et al., 1994). Chemoreception is not only used in the detection of prey, but also to enable the larvae to stay in the vicinity of the prey (Knutsen, 1992). This is particularly important in the wild, where zooplankton often occurs in patchy distribution, and mortality is commonly associated with a failure of the fish larvae to capture sufficient and/or appropriate prey items (Houde, 1978; May, 1974). Tanaka et al. (1991) suggest yolk sac larvae of the red sea bream, *Pagrus major*, use chemoreception to actively associate with areas of high prey density in order to minimise energy consumption locating prey at first-feeding. Habitat type is closely associated with presence of olfactory receptors, and in general demersal fish larvae hatch with olfactory receptors present, whereas receptors in pelagic larvae develop later and are commonly observed post-hatching (Kjørsvik et al., 2004). Demersal fish often inhabit

low-light environments where feeding is more reliant on the non-visual senses (Jones and Janssen, 1992; Kasumyan, 2011). While larvae of different species display varying timing in olfactory development, it has been shown that in many species the olfactory response to predators matures earlier than the olfactory response to prey, indicating the importance of chemoreception in predator avoidance (Kasumyan, 2011). In most fish the gustatory system is more sensitive than the olfactory system (Kasumyan and Døving, 2003), with food search behaviour driven by the olfactory system and completed by the gustatory system (Hara, 2006). The taste buds are composed of elongated sensory cells that appear like the segments of an orange and are generally first observed just prior to first-feeding (Kasumyan, 2011; Lagler et al., 1962). Taste buds may be classed as intraoral or extraoral, with the extraoral system developing earlier and having greater sensitivity and response to a wider range of stimuli than the intraoral system (Kasumyan, 2011).

1.8 First-feeding and the visual environment

Broadcast spawning fish produce large numbers of small eggs that hatch as small larvae (Bone et al., 1995). These larvae are in an undeveloped state, have eyes incapable of image formation, possess poor locomotory ability and are reliant on endogenous yolk reserves for their energy requirements (Bone et al., 1995; Werner, 2002). Fuiman (1988), cited in Kotrschal et al. (1990), suggest that the high mortality of broadcast spawning progeny is associated with insufficient sensory capability of the undeveloped larvae, which limits the detection of prey and predators. As most fish larvae are visual feeders, the transition from endogenous to exogenous feeding requires rapid morphological development of the sensory apparatus so that the larvae have the ability to locate and capture prey (Batty and Hoyt, 1995; Blaxter, 1986). This is a critical phase in larval development where the consequence of failing to capture sufficient prey, as a result of limited visual ability, is mortality due to starvation (Blaxter, 1986; Kotrschal, et al., 1990; Hjort, 1914; May, 1974).

Fish live in diverse aquatic habitats characterised by differences in a number of factors, including light intensity, spectral quality, concentration of primary productivity and turbulence induced by currents and winds (Costello, 2009; Jerlov, 1976). As larvae display visual adaptations that reflect their specific visual habitat (Guthrie and Muntz, 1993), abiotic and biotic factors can greatly affect the ability of larvae to perceive prey. The aquaculture environment allows the manipulation of abiotic and biotic factors such as light intensity, turbidity, tank colour, turbulence, prey density, prey size and larval density, consequently, the culture conditions that promote best feeding can be provided once the larval requirements are defined. The provision of optimum first-feeding parameters in culture leads to reduction in early mortality and improvement in health, growth and viability of larvae during their early life history (Downing and Litvak, 2001; Naas et al., 1992; Parra and Yufera, 2000).

Light is the environmental factor which has the greatest impact on feeding in most marine fish, with the threshold intensity for feeding being species-specific (Blaxter, 1986; Carton, 2005; Cobcroft, 2001; Downing and Litvak, 2001; Pankhurst and Hilder, 1998; Villamizar et al., 2011b). While larvae are capable of feeding within a given light intensity range, identification of the optimum light level significantly improves larval performance in terms of growth and survival. On the other hand, sub-optimal light levels have been linked with malformation incidence and poor swimbladder inflation (Blaxter, 1968; Downing and Litvak, 1999; Stuart and Drawbridge, 2011; Woolley et al., 2012a). The use of algae (green water) in larviculture has been documented to improve larval performance in a number of ways including enhanced nutrition of live prey, diffuse scattering of light reducing walling behaviour, increased prey detection by providing a greater prey contrast against the background, and water conditioning (Cobcroft and Battaglene, 2009; Miner and Stein, 1993; Naas et al., 1992; Shaw, 2006; Utne-Palm, 2002). Tank colour provides contrast of prey against background colour, but it also affects the distribution of light within the culture environment through reflection and scattering. It may also match the spectral sensitivity of the larvae (Cobcroft and Battaglene,

2009; Monk et al., 2008; Naas et al., 1996; Strand et al., 2007; Tamazouzt et al., 2000). Higher turbulence levels in culture have the potential to increase the rate of predator-prey interactions providing the larvae with a greater opportunity for successful feeding (Stiansen and Sundby, 2001), although turbulence levels should not exceed the ability of the larvae to detect, pursue and capture prey. Increasing prey density also has the ability to improve larval feeding by providing an increase in predatory encounter rate, although, feeding to excess has the potential to pollute the aquaculture environment and waste economic resources (Houde and Schekter, 1980; Robert et al., 2009; Shoji and Tanaka, 2004). The relationship between prey density and feeding response is known to vary among species and developmental stage (Dou et al., 2000; Parra and Yufera, 2000; Shaw, 2006). In addition to prey density, the size of the prey must be ingestible by the larvae as they are gape-limited predators (Puvanendran et al., 2004). Larvae are known to consume zooplankton prey items as large as their mouth gape will allow, which benefits the larvae in terms of enhanced energy intake, increasing the probability of survival (Anto et al., 2009; Graham and Sprules, 1992). Correct larval stocking density should provide the highest yield from the culture tank in terms of the number and quality of seed, while also encompassing the fastest rate of production (Hitzfelder et al., 2006; King et al., 2000). Plasticity in the aquaculture environment allows the manipulation of culture variables in order to meet species-specific requirements.

Recent studies have documented the importance of high light intensity and turbidity in the culture of *S. lalandi* larvae (Carton, 2005; Stuart & Drawbridge, 2011; Woolley, et al., 2012a), although information regarding the requirements of *T. maccoyii* larvae is much more limited (Hutchinson, 2009; Cobcroft, et al., 2012b). Many of the culture parameters for *T. maccoyii* and *S. lalandi* implemented at Clean Seas Tuna Ltd have been determined through empirical evidence (Table 5). The novel nature of *T. maccoyii* larviculture, in addition to high larval mortality, has restricted the identification of optimum larviculture conditions and consequently requires further elucidation.

Table 5. Culture parameters used at Clean Seas Tuna Ltd Arno Bay SA.

Culture parameter	*Thunnus maccoyii*	*Seriola lalandi*
Temperature	26^0C	24^0C
Salinity	36 ‰	36 ‰
Dissolved oxygen	> 100%	> 100%
pH	8.0	7.8
Light Intensity	50 µmol s^{-1} m^{-2} *	126 to 203 µmol s^{-1} m^{-2} **
Turbidity	1 to 2 NTU	1 to 1.5 NTU
Photoperiod	14 :10 (h L: D)	14 :10 (h L: D)
Turbulence	Water induced up-welling	Air induced up-welling
Rotifers	10 mL^{-1} first feeding	5 mL^{-1} first feeding
Artemia	0.1 mL^{-1} > 11 dph	0.1 mL^{-1} > 10 dph
Newly hatched larvae	0.5 L^{-1} > 15 dph	Nil

*Large variation in ambient sunlight levels (natural sunlight recorded levels >766 µmol s^{-1} m^{-2} entering the larviculture tanks). **Converted from lx using Thimijan and Heins (1982). Information obtained from Cobcroft, et al., 2012b; Cobcroft, 2013 and B. Chen pers. comm. at CST Ltd.

1.9 The underwater light environment

Fish eyes are adapted to the aquatic environment in response to the physics of light (Brett, 1957; Job and Bellwood, 2000). Sunlight is comprised of photons in the visible wavelengths (380 to 780 nm) as well as the spectrum including the infrared (> 700 nm) and ultraviolet (< 380 nm) wavelengths (Jobling, 1995). As light passes through water, the liquid media acts like a chromatic filter selectively absorbing different spectra (colours) (Villamizar et al., 2009). Spectral bandwidth and intensity are rapidly reduced with increasing depth (Jerlov, 1976). Short wavelengths less than 390 nm (violet light) and long wavelengths greater than 600 nm (red light) are rapidly absorbed compared to wavelengths of 450 nm (blue light) (Villamizar et al., 2011a). Apart from shallow or turbid conditions, the predominant spectral transmission of light in water is in the blue spectrum. Due to the diversity of the aquatic environment, changes in spectral composition and intensity are not only observed vertically but also horizontally (Jerlov, 1976). Eutrophic waters with high particulate loads exhibit rapid scatter and absorption of light and are characterised by maximum light transmission in the green-yellow spectrum (Job and

Shand, 2001). In contrast, oligotrophic waters with a low particulate load are typified by maximum light transmission in the blue spectrum (Jerlov, 1976).

1.10 The visual pigments

The ability of fish to see is ultimately dependent on the capture of photons by the visual pigments located in the photoreceptors. Visual pigments are characterised by having a maximum absorption at a specific wavelength (λ_{max}) which determine the wavelength ('colour') of light to which a fish is most sensitive (Utne-Palm and Bowmaker, 2006). The spectral sensitivity of the visual pigments is dependent on the amino acid sequence of the opsin protein and whether this is linked to a chromophore derived from vitamin A1 (rhodopsin) or vitamin A2 (prophyropsin) (Bowmaker, 1995). Photoreceptors in fish exhibit a number of morphological differences including single, twin, triple and quadruple cones, and exhibit a broad range of spectral sensitivities. The two photoreceptors forming the twin cones may display differences as they may be morphometrically identical and express the same visual pigment or morphometrically identical and express different visual pigments, or be different in form and visual pigment.

The different spectral peak sensitivities (λ_{max}) expressed by the photoreceptors allow 'spectral adjustment' by the fish in response to light conditions (Bowmaker, 1995). Studies have shown species-specific visual pigment λ_{max} (particularly twin cones) reflecting the spectral transmission of light in the fish's natural environment (Bowmaker, 1990; Loew and Lythgoe, 1978; Loew et al., 2002; Shand, 1993; Shand et al., 2002). For example, deep-sea fish possess a narrow spectral range between 470-480 nm that corresponds with the transmission of light found in deep oceanic water (Bone et al., 1995; Shand, 1993), whereas fish inhabiting shallow coastal waters exposed to a relatively broad spectral bandwidth, predominantly have a peak transmission in the green range (Jerlov, 1976), and a spectral sensitivity ranging from 490 to 575 nm (Bone et al., 1995; Britt et al., 2001). Plasticity in the ability to alter spectral sensitivity has

been observed in fish at different developmental ages, as fish move from one optical environment to another (Bone et al., 1995; Shand et al., 2002). Changes in spectral sensitivity have been recorded in surface dwelling black bream, *Acanthopagrus butcheri* larvae settling in deeper waters during and after metamorphosis, and in migratory salmonids moving between freshwater and seawater, (Bone et al., 1995; Shand et al., 2002).

The visual performance of larval fish can be optimized in optical environments that best match their particular ecological niche (Downing and Litvak, 2001). Identification of visual pigment λ_{max} for species under culture investigation is therefore a significant tool in understanding the visual adaptations of larvae in the wild, which may in turn have significant culture implications (Miyazaki et al., 2008).

1.11 Animal ethics

My study was conducted with approval from the University of Tasmania Animal Ethics Committee, approval number A0010990, complying with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes – 7th edition, 2004.

1.12 Thesis structure

The thesis is structured with a general introduction, four research chapters and a final discussion. All data chapters (2, 3, 4 and 5) are presented in journal format and have been submitted or are being submitted for publication in Aquaculture and Aquaculture Research. There is some unavoidable repetition in the introduction, materials and methods, results, discussion and reference sections.

Chapter 1 provides a general background on concepts discussed throughout the thesis while introducing the scope of the study and outlining the aims. Chapters 2 and 3 investigate the effect the visual environment has on the first-feeding response of *T. maccoyii* and *S. lalandi*. Larvae were studied in short-term, small scale experiments where their feeding was tested in response to a range of factors including; light

intensity, turbidity, tank colour, turbulence, prey density, prey size and larval density. The intention was to identify parameters, both positive and negative, that significantly affected first-feeding. Chapter 4 investigates the effect of light intensity and prey density (factors identified in Chapters 2 and 3 as important and requiring further research) with increasing larval age, 3, 6 and 9 dph, in similar behavioural feeding experiments to Chapters 2 and 3. The evaluation of feeding response with age was identified as important due to the rapid morphological changes larvae display with increased growth. Chapter 5 investigates the retinal morphology and sensitivity of both species in order to identify developmental sequences and species-specific retinal adaptations. These were used to help explain the observed feeding responses and better elucidate larval requirements during early culture. In Chapter 6 the key findings from Chapter 2 through to Chapter 5 are discussed in relation to the requirements for culture and possible ecological considerations.

1.13 References

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Chapter 2. A comparison between the first-feeding response of southern bluefin tuna, *Thunnus maccoyii*, and yellowtail kingfish, *Seriola lalandi*, larvae to prey density, prey size and larval density

2.1 Abstract

Initiation of first-feeding is critical to the survival of marine finfish larvae. I investigated the first-feeding success of two species: southern bluefin tuna, *Thunnus maccoyii*, and yellowtail kingfish, *Seriola lalandi*. I examined feeding performance (proportion and intensity) in three short-duration (4 h) experiments i.e., prey density (0.5 to 25 rotifers mL^{-1}), prey size (small and large rotifers at densities of 2, 5 and 25 rotifers mL^{-1}), and larval density (2 to 75 larvae L^{-1}). Increasing prey density significantly increased the proportion of larvae feeding but not feeding intensity in both species. Prey size alone did not significantly affect the proportion or intensity of feeding for either species. *Seriola lalandi* exhibited a significant decrease in the proportion of larvae feeding with increasing larval density concurrent with a significant increase in feeding intensity. In *T. maccoyii*, larval density had a significant effect on the proportion of larvae feeding. The overall feeding performance amongst all experiments was higher in *T. maccoyii* (maximum feeding response 83% and 0.032 rotifers $larva^{-1}$ min^{-1}) than *S. lalandi* (23% and 0.022 rotifers $larva^{-1}$ min^{-1}). The results from my study have identified important biotic factors that affect the first-feeding ability of *T. maccoyii* and *S. lalandi* larvae. This information has the potential to improve the first-feeding success with subsequent improvement in the culture of both species.

2.2 Introduction

Southern bluefin tuna, *Thunnus maccoyii*, produced from domestically held broodstock is under investigation as a new species to aquaculture in Australia (Thomson et al., 2010). Due to the early stage of industry development, culture requirements for this species are not yet fully understood and high early larval mortalities have slowed commercialisation. Research is constrained by the relatively short spawning season of *T. maccoyii*, which is approximately 10 weeks in duration (Cobcroft et al., 2012). Major mortality is commonly seen in larval culture among the tuna species (Davis et al., 1991; Fukuda et al., 2010; Ishibashi et al., 2009; Kaji et al., 1996; Margulies, 1997; Sawada et al., 2005). As a result, there is a limited understanding of the basic larval physiological requirements of many tuna species (Fukuda et al., 2010; Kawamura et al., 2003; Matsumoto et al., 2009), which limits the transfer of successful culture technology within the genus.

My study of the first-feeding capacities of *T. maccoyii* and yellowtail kingfish, *Seriola lalandi*, investigated the feeding performance of both species in order to compare their similarities and differences with a view to gaining a greater understanding of larval requirements and contribute to the future larviculture of both species. *Thunnus maccoyii* and *S. lalandi* are broadcast spawners; their larvae are oceanic and planktonic with similar larval morphology, and exhibit fast growth. Several studies have described rearing conditions for *S. lalandi* (Benetti et al., 2005; Carton and Vaughan, 2010; Chen et al., 2006; Chen et al., 2007; Cobcroft, 2013; Hilton et al., 2008; Moran et al., 2011; Woolley et al., 2012) and there are established aquaculture industries in Australia, Japan and New Zealand (Benetti et al., 2005; Kolkovski and Sakakura, 2004; Nakabo, 1993; Nakada, 2000; Poortenaar et al., 2000).

In order to successfully culture fish, it is pivotal to gain a thorough understanding of early-larval development and larval requirements. First-feeding is a critical phase in the larviculture of marine finfish, with the transition from endogenous to exogenous feeding often resulting in high

mortality (Blaxter, 1988). Mortality is commonly associated with a failure of the larvae to capture sufficient and/or appropriate prey items (May, 1974). Fish possess a number of senses that allow the detection and palatability of prey including vision, mechanoreception, olfaction and gustation, however, vision is considered the primary sense required for successful feeding of many marine finfish larvae (Batty and Hoyt, 1995; Blaxter, 1986). Since many larvae initiate feeding with developing visual apparatus (Blaxter and Jones, 1967; Cobcroft and Pankhurst, 2003), several biotic factors in the visual environment, such as prey density, prey size and larval density, can impact on the ability of first-feeding larvae to identify and capture prey items (Hecht et al., 1996; Houde, 1975; 1978; Hunter, 1980; Shaw et al., 2003). The density of prey is critically important, affecting the first-feeding success of larvae of a number of species, as it directly influences the likelihood of prey encounter, which is restricted by a visual limit of around one larval body length (Blaxter, 1986; Houde and Schekter, 1980; Robert et al., 2009; Shoji and Tanaka, 2004; Slembrouck et al., 2009). With developing visual apparatus and limited swimming and feeding ability, an increase in the prey encounter rate increases the chance for prey capture (Shoji and Tanaka, 2004). In addition to prey density, the prey size offered is also important as larval fish are gape-limited predators and prey size must permit physical ingestion (Puvanendran et al., 2004). Optimal larval densities should provide the highest yield from culture tanks at the fastest rate of production, while also providing the best economic returns (King et al., 2000). In a controlled environment, such as a larval-rearing system, many of these factors can be manipulated to maximise first-feeding success and minimise mortality associated with inadequate early feeding.

My study investigated the feeding performance of *T. maccoyii* and *S. lalandi* exposed to different visual predator-prey conditions such as prey density, prey size and larval density. My aim was to test the species over a wide range of conditions, in order to identify selected biotic factors commonly encountered during the culture of fish larvae that influence the

first-feeding success for both species, leading to a better understanding of their larval rearing requirements.

2.3 Materials and methods

2.3.1 Embryo supply and rearing

Thunnus maccoyii and *S. lalandi* embryos were supplied by Clean Seas Tuna Ltd from their hatchery facility in Arno Bay, South Australia. *Thunnus maccoyii* embryos were collected from four spawning events during February 2010, and *S. lalandi* embryos were collected from three spawning events during February and March 2011. Immediately after spawning the embryos were collected from the broodstock tank and assessed for viability. Fertilisation was greater than 80% for all cohorts.

Significant demand for *T. maccoyii* embryos during the relatively short spawning season, to conduct research and commercial-scale trials, resulted in a restricted number of viable *T. maccoyii* embryos for feeding experiments. As such, *T. maccoyii* embryos were unavailable for direct experimental comparison with *S. lalandi* in 2011, and consequently experiments were conducted in 2010 for *T. maccoyii* and 2011 for *S. lalandi*. Culture parameters for *T. maccoyii* and *S. lalandi* embryos, prior to hatch, are summarised in Table 1A. Each embryo cohort was reared in an individual tank.

At hatch, *T. maccoyii* embryos remained in the same rearing tank due to the relatively low larval density of 0.5 larvae L^{-1}. The tank base colour differed in one of the three cohorts of embryos reared (i.e., one white-based and two green-based), although as the embryos had non-functional eyes until the beginning of day 3, it is highly unlikely to have affected the larvae.

At hatch, *S. lalandi* embryos were transferred to a different 2500 L green fibreglass, cylindro-conical larviculture tank (Table 1B). The transfer protocol involved removing the air from the incubator tank to concentrate the positively buoyant larvae at the surface. Larvae were then collected in

3 L beakers and transferred into larviculture tanks until a stocking density of 10 ± 2 larvae L^{-1} was achieved.

Table 1. Culture parameters for *Thunnus maccoyii* and *Seriola lalandi* embryos A) prior to hatching and B) post-hatching.

A)

Culture parameter	*Thunnus maccoyii*	*Seriola lalandi*
Embryo disinfection	0.53 ppm ozone (1 min)	Nil
Time to hatch	26 h @ 25 ^{0}C	50 h @ 20 ^{0}C
Fibreglass tank	2500 L cylindro-conical	2500 L cylindro-conical
Tank colour	Green only or green with white base	White
System	Flow-through	Flow-through
Water treatment	1 µm filtered, UV treated	1 µm filtered, UV treated
Salinity	36 ± 0.2 ‰	36 ± 0.2 ‰
Water temperature	26.0 ± 1.0 ^{0}C	24.0 ± 0.7 ^{0}C
pH	8.2 ± 0.1	7.8 ± 0.3
Dissolved oxygen	98.0 ± 1.0%	97.5 ± 1.7%
Flow rate	416 L h^{-1} (i.e., 400% exchange per day)	1800 L h^{-1} (i.e., 1800% exchange per day
Photoperiod	12:12 (h L: D)	14:10 (h L: D)
Light intensity (max)	0.38 µmol s^{-1} m^{-2}	12 µmol s^{-1} m^{-2}
Stocking density	10 ± 2 eggs L^{-1}	170 ± 46 eggs L^{-1}
Water current	Upwelling water or single open-ended air line	Single open-ended air line

B)

Culture parameter	*Thunnus maccoyii*	*Seriola lalandi*
Fibreglass tank	As above	As above
Tank colour	As above	Green
System	As above	As above
Water treatment	As above	As above
Salinity	As above	As above
Water temperature	26.0 ± 0.5 ^{0}C	23.8± 1.0 ^{0}C
pH	8.1 ± 0.1	7.88± 0.1
Dissolved oxygen	95.8 ± 1.2%	97.2 ± 1.6%
Flow rate	625 L h^{-1} (i.e., 600% exchange per day)	416 L h^{-1} (i.e., 400% exchange per day)
Photoperiod	As above	As above
Light intensity (max)	As above	0.38 µmol s^{-1} m^{-2}
Stocking density	10 ± 2 larvae L^{-1}	10 ± 2 larvae L^{-1}
Water current	As above	As above

Seriola lalandi larval behaviour late on 2 dph indicated a proportion of larvae were ready to commence feeding. Feeding experiments were attempted on 2 dph although poor or nil feeding was observed. The low feeding response in the experimental system and a proportion of larvae not feeding in the larval source tank indicated the cohort as a whole were not ready to commence feeding. In order to not disadvantage the proportion of larvae that were ready to feed, rotifers were added to the larval source tank on 2 dph at 10 mL^{-1} (enriched with Spresso® INVE, Belgium), a turbidity of 3 nephelometric turbidity units (NTU) was induced by algal paste (Nanno 3600®, Reed Mariculture, California) and a light intensity of 24 µmol s^{-1} m^{-2} (at the tank surface in the centre of the tank) was provided by a metal halide light (Phillips 400 W). While a proportion of larvae initiated feeding on 2 dph, first-feeding was defined as 3 dph.

2.3.2 Stocking of experimental system

Feeding experiments were conducted on *T. maccoyii* and *S. lalandi* on the first day of feeding (3 dph) (Fig. 1). To enable the capture of larvae from the larviculture source tank, the light level was increased to 24 µmol s^{-1} m^{-2} (Phillips 400 W, metal halide). Approximately 30 larvae were siphoned via a 10 mm Ø plastic tube into a 250 mL beaker and transferred to each 3 L aquaria. A white circular disc (20 cm Ø) attached to an extension arm was submerged in the larviculture tank to aid in capture by providing a background to highlight the larvae.

The experimental set-up consisted of 24 individual, 3 L black, hemispherical, plastic aquaria. The aquaria were randomly allocated to one of six treatments with each treatment having four replicates. Light was provided by a single fluorescent tube (NEC tri-phosphor 18 watt, FL20SSBR/ 18-HG, T8) suspended above each treatment group of aquaria, providing a light intensity of 30 ± 2 µmol s^{-1} m^{-2} (n = 6), range 28 - 33 µmol s^{-1} m^{-2}. The aquaria were maintained as static systems, with no aeration and no water flow.

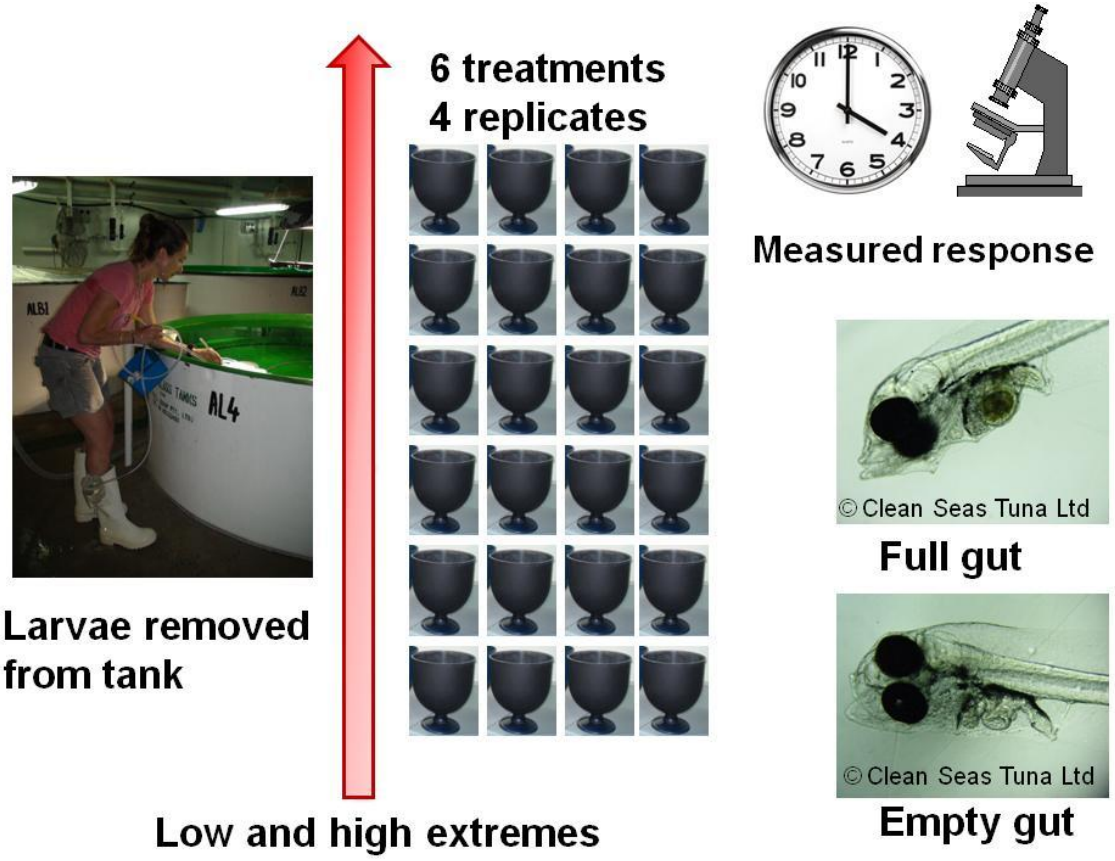

Figure 1. Schematic diagram showing an overview of the feeding experiments. Larvae were caught from the larviculture source tank, designated to a treatment and left to feed for 4 hours prior to evaluation as feeding or not feeding.

Each feeding experiment was run over a 4 h feeding period. At least 30 minutes was allowed for acclimation prior to the addition of rotifers, initiating the start of the experiment, and the lights were on during acclimation. A rotifer density of 2 mL^{-1} for each aquaria was chosen (unless otherwise stated) in order to challenge the larvae to feed. It was important to provide a rotifer density that allowed significant feeding differences to be observed between the treatments, while not allowing feeding to satiation or totally inhibiting feeding.

In order to provide the same feeding period (4 h) to each aquarium throughout the experiment, rotifers were added to one aquarium at a time, with a 3 minute interval between each addition. This allowed a 3 minute time frame to collect larvae from each aquarium prior to sampling the following aquarium (24 aquaria in total) (Cobcroft et al., 2001). Larvae

were euthanised by anaesthetic (0.06% AQUI-S ®, New Zealand Ltd) at the end of each experiment. Standard length (SL) ± standard deviation (sd) (here and throughout), and eye diameter ± sd (n = 20) were recorded for all sampled larvae on the day of the experiment.

2.3.3 Evaluation of feeding response and mortality

Assessment of feeding response involved “squashing” the euthanised larvae with a cover slip on a microscope slide resulting in the rupture of the stomach wall enabling the presence/absence of rotifers to be determined. Prior examination revealed *T. maccoyii* and *S. lalandi* larvae did not regurgitate food upon euthanasia. Individual rotifer mastax were counted to record the number of rotifers consumed per larva. The proportion of feeding, intensity of feeding (rotifers larva^{-1} min^{-1}) and mortality was recorded as mean ± sd (n = 4).

The proportion (%) of larvae feeding was calculated from the following equation:

Proportion feeding = feeding larvae / live larvae x 100

Feeding intensity was expressed as rotifers larva^{-1} min^{-1} and was calculated as:

Feeding intensity = rotifers ingested per feeding larva / 240 min

Where 240 min represents the experimental feeding time.

The mortality (%) was calculated as:

Mortality = dead larvae / total number of larvae x 100

The 4 h feeding duration was too short for total decomposition of dead larvae to occur, so an accurate assessment of mortality was obtained.

2.3.4 Feeding experiments

The response of *T. maccoyii* and *S. lalandi* to prey density, prey size and larval density was investigated. The range of variables tested within each treatment extended above and below standard culture parameters

commonly used in aquaculture. This allowed standard culture parameters to be tested, while also evaluating larval feeding response against the lower and higher ranges of culture increasing the potential to define optimum rearing conditions.

2.3.4.1 Prey density and first-feeding

Six different prey densities were tested which included; 0.5, 1, 2, 5, 15 and 25 rotifers mL^{-1}.

2.3.4.2 Prey size, density and first-feeding

This multi-factorial experiment tested prey density in conjunction with rotifer size. Large or small rotifers were fed at densities of 0.5, 2 and 25 mL^{-1}. Rotifer sizes are reported in section 2.3.5.

2.3.4.3 Larval density and first-feeding

Thunnus maccoyii were stocked at 2, 5, 7, 20, 40 and 65 larvae L^{-1} and *S. lalandi* were stocked at 2, 5, 10, 25, 50 and 75 larvae L^{-1}. Rotifer density was maintained at 2 mL^{-1} for all larval density treatments.

2.3.4.4 Surface oil and first-feeding

This experiment tested the response of *T. maccoyii* (n = 30) in four aquaria with and four without, the addition of a drop of squid oil, at a light intensity of 30 µmol s^{-1} m^{-2} in order to reduce surface tension and test coincident effects on feeding and mortality.

2.3.5 Rotifers

Large-strain (L-S) rotifers (*Brachionus plicatilis*) and small-strain (S-S) rotifers (*Brachionus rotundiformis*) were used in the 2010 season (N.B. S-S rotifers were only used in the prey size feeding experiments). L-S rotifers had a length of 240 ± 21 µm (n = 30) compared to S-S rotifers with a length of 150 ± 10 µm (n = 30). Rotifer length was significantly different ($F_{1, 58} = 310.718$, $P < 0.001$).

Rotifer volume was calculated using the equation for an ellipsoid:

$$Rotifer\ volume = 4/3\ \pi ab^2$$

Where *b* (rotifer width) is assumed to be half of *a* (rotifer length).

Rotifer volume of L-S was 14.4 x 10^6 µm^3, and S-S rotifers had a volume of 3.5 x 10^6 µm^3. Small-strain rotifers were unavailable in the 2011 season, so L-S rotifers were screened twice through a 120 µm bag screen to collect rotifers classed as large (244 ± 26.6 µm length, n = 30) and small (190 ± 24.4 µm length, n = 30). Rotifer lengths were significantly less in small rotifers than large rotifers ($F_{1, 58} = 66.996$, $P < 0.001$). The volume of large rotifers was 15.2 x 10^6 µm^3, while small rotifers had a volume of 7.1 x 10^6 µm^3.

2.3.6 Assessment of transfer stress from the larviculture source tank to the experimental system

The feeding response of larvae in the larviculture source tank and larvae that had been transferred into the experimental system was compared to evaluate the effect of transfer stress. Similar feeding conditions were provided to the larviculture source tank as the experimental system, which consisted of a turbidity of 3 NTU achieved through the addition of Nanno 3600®, rotifers added at a density of 2 mL^{-1} and a light intensity of 24 µmol s^{-1} m^{-2} at the tank surface in the centre of the tank. Rotifers were enriched with Spirit ® INVE, Belgium and Nanno 3600 ® for *T. maccoyii* and Spresso® for *S. lalandi*. Larvae were left undisturbed for the same 4 h feeding period as the experimental aquaria. The proportion of larvae feeding was recorded for 20 larvae per larviculture source tank.

2.3.7 Statistics

The one-factorial prey density and larval density experiments, on the proportion of fish feeding and mortality, were analysed using chi-square analysis (SPSS statistics 19, IBM) as the data were dichotomous (i.e., feeding or not feeding and either dead or alive). Standardised residuals ≥ 2 and ≤ -2 identified significant differences within the treatments and the

observed results were reported as more than expected or less than expected (chi-square tests the difference between the observed results and the hypothetical distribution that may be expected due to chance or probability). Graphs were depicted as proportion (%) to allow easy interpretation. The data were presented as mean + sd (four replicates per treatment). Intensity of feeding was analysed by a nested one-way ANOVA. A binomial logistic regression analysis was conducted in R studio 0.97.246 – Windows xp/Vista/7 (*R Development Core Team (2011) R A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/.*) to determine the effect of prey size and prey density on the proportion of larvae feeding. Analyses of deviance validated the model fit. To improve model fit, non-significant variables were removed from the analysis and the model re-fitted. Feeding intensity in the prey size and prey density experiment was analysed by two-way ANOVA. Data were evaluated for homogeneity of variance using Levene's test. Tukey's post hoc test was used to describe differences between means when the ANOVA was significant. Statistical significance accepted at $P \leq 0.05$.

2.4 Results

2.4.1 Larval standard length and eye size

On the day of the feeding experiment (3 dph) the standard length of *T. maccoyii* was 3.13 ± 0.10 mm and eye diameter was 0.241 ± 0.010 mm. Larvae had a relative eye size of 7.8% of standard length and no difference in standard length or eye diameter was observed between cohorts ($F_{2, 57} = 0.580$, $P = 0.563$ and $F_{2, 57} = 1.641$, $P = 0.203$, respectively). In comparison, 3 dph *S. lalandi* larvae had a standard length of 4.54 ± 0.08 mm and eye diameter of 0.343 ± 0.003 mm. The relative eye size of *S. lalandi* was 7.6% and no significant difference in standard length or eye diameter was observed between cohorts ($F_{2, 57} = 1.773$, $P = 0.179$ and $F_{2, 57} = 0.523$, $P = 0.881$, respectively).

2.4.2 Feeding experiments

2.4.2.1 Prey density and first-feeding

Increasing prey density significantly increased the proportion of *T. maccoyii* larvae feeding ($\chi^2 = 29.280$, df 5, $P < 0.001$) with fewer feeding responses occurring at the lowest prey density (0.5 mL^{-1}) and more feeding responses occurring at the highest prey density (25 mL^{-1}) (Fig. 2A). In contrast, there was no significant difference in the proportion of *S. lalandi* larvae feeding ($\chi^2 = 7.016$, df 5, $P = 0.219$) with changing prey density, although a gradual increase was observed as prey density increased (6.4 ± 4.6% increasing to 14.1 ± 4.7%) (Fig. 2C). The proportion of *T. maccoyii* feeding was five times higher than *S. lalandi* (Table 2).

The feeding intensity with increasing prey density was not significantly different for either *T. maccoyii* ($F_{5, 18} = 1.234$, $P = 0.33$) (0.013 ± 0.005 rotifers $larva^{-1}$ min^{-1}, Fig. 2B) or *S. lalandi* ($F_{5, 18} = 1.632$, $P = 0.202$) (0.006 ± 0.002 rotifers $larva^{-1}$ min^{-1}, Fig. 2D) although the feeding intensity was three times greater in *T. maccoyii* than *S. lalandi*.

Mortality of *T. maccoyii* was high, ranging from 33 to 58%, and was significantly affected by prey density, with greater than expected survival in the 2 rotifers mL^{-1} treatment ($\chi^2 = 12.651$, df 5, $P = 0.027$). Larvae in the remaining prey density treatments all recorded similar mortalities. *Seriola lalandi* showed no significant difference in mortality among prey density treatments ($\chi^2 = 1.315$, df 5, $P = 0.933$).

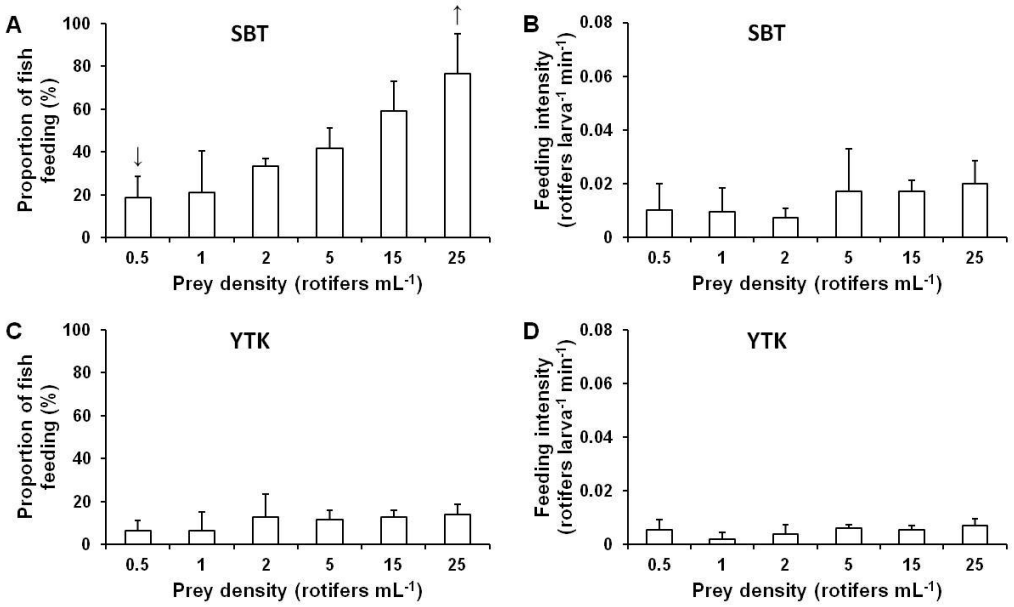

Figure 2. First-feeding response of fish larvae fed different prey densities, in *Thunnus maccoyii* (SBT) (A) the proportion feeding and (B) feeding intensity, and in *Seriola lalandi* (YTK) (C) the proportion feeding and (D) feeding intensity. The arrows indicate treatments in which there were significantly more (↑) or less (↓) larvae feeding than expected (chi-square; $P \leq 0.05$). Mean + sd, n = 4.

Table 2. Summary of the proportion feeding (%), feeding intensity (rotifers $larva^{-1}min^{-1}$) and mortality data (%) for *Thunnus maccoyii* and *Seriola lalandi*.

Experiment	**Parameter**	**Treatment range**	
		T. maccoyii	***S. lalandi***
Prey density	Proportion feeding	18 - 77	6 - 14
	Feeding intensity	0.007 - 0.020	0.002 - 0.007
	Mortality	33 - 58	3 - 20
Prey size and density	Proportion feeding	45 - 90	10 - 23
	Feeding intensity	0.013 - 0.027	0.0002 - 0.001
	Mortality	32 - 49	0 - 13
Larval density	Proportion feeding	63 - 82	13 - 34
	Feeding intensity	0.035 - 0.049	0.001 - 0.060
	Mortality	0 - 17	0 - 20

2.4.2.2 Prey size, density and first-feeding

Based on the logistic regression analysis (Table 3), prey density had a significant effect on the proportion of *T. maccoyii* feeding (Fig. 3A), although prey size, and any interaction between prey size and density was not significant. The likelihood of *T. maccoyii* feeding in the 25 mL^{-1}

treatment was 2.07 to 6.23 times higher than feeding in the 0.5 mL^{-1} and 2 mL^{-1} treatment. The proportion of *S. lalandi* larvae feeding also significantly improved with increasing prey density (Table 3). The likelihood of feeding in the 2 mL^{-1} and 25 mL^{-1} treatments was 1.15 to 3.39 and 1.30 and 3.76 times higher respectively, than feeding in the 0.5 mL^{-1} treatment (Fig. 3C). The proportion of *T. maccoyii* feeding was four times higher than *S. lalandi.*

No effect of prey size or any interaction between prey size and prey density was observed in the proportion of *S. lalandi* larvae feeding. *Thunnus maccoyii* feeding intensity (analysed by two-way ANOVA) significantly improved with increasing prey density and an interaction between high prey density (25 mL^{-1}) and S-S rotifers significantly increased feeding intensity (Table 4). The feeding intensity of *T. maccoyii* was 27 times higher than *S. lalandi*.

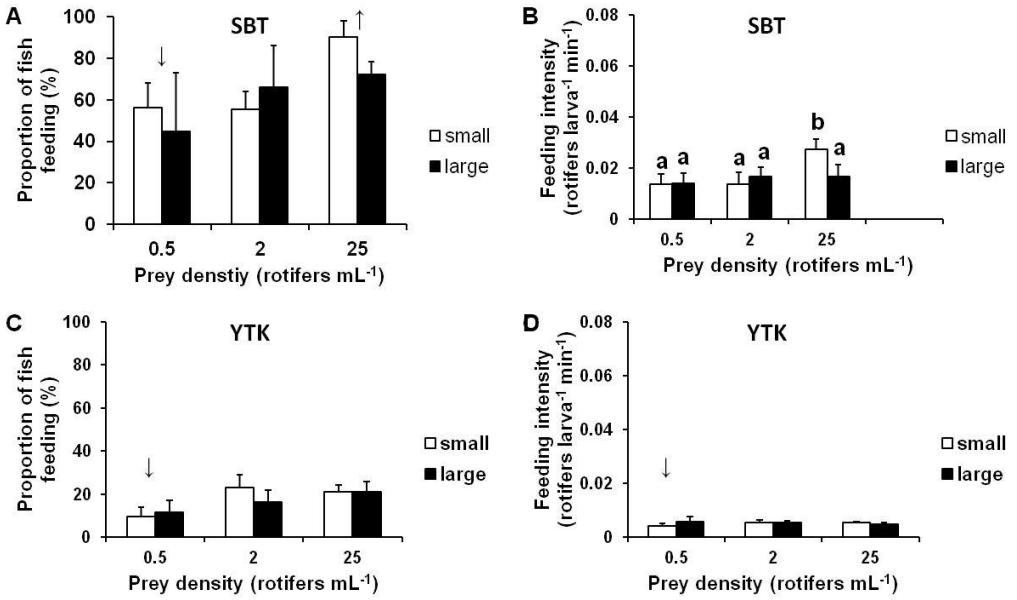

Figure 3. First-feeding response of fish larvae fed rotifer prey of two different sizes (small and large) and three different prey densities. In *Thunnus maccoyii* (SBT*)* larvae (A) the proportion feeding and (B) feeding intensity, and in *Seriola lalandi* (YTK) larvae (C) the proportion feeding and (D) feeding intensity. The arrows indicate those categories in which there were more (↑) or less (↓) larvae feeding than expected (chi-square; $P \leq 0.05$). Means sharing a common letter are not significantly different. Mean + sd, n = 4.

Table 3. Statistical summary for the multi-factorial prey size and prey density experiment, showing proportion feeding and mortality data (binomial logistic regression analysis) for *Thunnus maccoyii* (SBT) and *Seriola lalandi* (YTK).

	Full model		Re-fitted model	
	Proportion feeding	Mortality	Proportion feeding	Odds 2.5% - 97.5%
SBT prey density 2 mL^{-1}	P = 0.038	P = 0.259	P = 0.201	
SBT prey density 25 mL^{-1}	P = 0.005	P = 0.826	P < 0.001	2.1 to 6.2
SBT prey size small	P = 0.373	P = 0.531		
SBT 2 mL^{-1} x small	P = 0.095	P = 0.802		
SBT 25 mL^{-1} x small	P = 0.121	P = 0.065		
YTK prey density 2 mL^{-1}	P = 0.337	P = 0.758	P = 0.013	1.1 to 3.4
YTK prey density 25 mL^{-1}	P = 0.052	P = 1.000	P = 0.003	1.3 to 3.7
YTK prey size small	P = 0.667	P = 0.776	-	
YTK 2 mL^{-1} x small	P = 0.287	P = 0.474	-	
YTK 25 mL^{-1} x small	P = 0.806	P = 0.624	-	

Table 4. *Thunnus maccoyii* (SBT) and *Seriola lalandi* (YTK) summary for the multi-factorial prey size and prey density experiment. Intensity of feeding (two-way ANOVA).

Experiment	Feeding intensity	Analysis
SBT prey size and density	Prey size * prey density	$F_{2, 24} = 5.760$, P = 0.012
	Prey size	$F_{1, 24} = 1.888$, P = 0.186
	Prey density	$F_{2, 24} = 8.460$, P = 0.003
YTK prey size and density	Prey size * prey density	$F_{2, 24} = 2.618$, P = 0.100
	Prey size	$F_{1, 24} = 0.575$, P = 0.573
	Prey density	$F_{2, 24} = 0.941$, P = 0.345

Prey size alone had no effect on the intensity of feeding in *T. maccoyii* (Fig. 3B). In comparison, feeding intensity in *S. lalandi* was not affected by prey size, prey density or the interaction of prey size and prey density (Table 4, Fig. 3D). The mortality of both *T. maccoyii* and *S. lalandi* was not affected by prey size, prey density or the interaction between prey size and prey density.

2.4.2.3 Larval density and first-feeding

The proportion of *T. maccoyii* larvae feeding was significantly different between treatments, with a lower proportion feeding than expected at 40 larvae L^{-1} (χ^2 = 20.934, df 5, P = 0.001), however, graphical representation of the data indicated that this result was not biologically important as the feeding incidence at a density of 40 larvae L^{-1} was within the range of all other treatments, some of which had higher variance (Fig. 4A). *Seriola lalandi* also showed a significant difference in the proportion of larvae feeding (χ^2 = 29.986, df 5, P < 0.001), with higher than expected feeding responses at larval densities of 2 and 5 larvae L^{-1} and a lower than expected feeding response at 50 larvae L^{-1} (Fig. 4C).

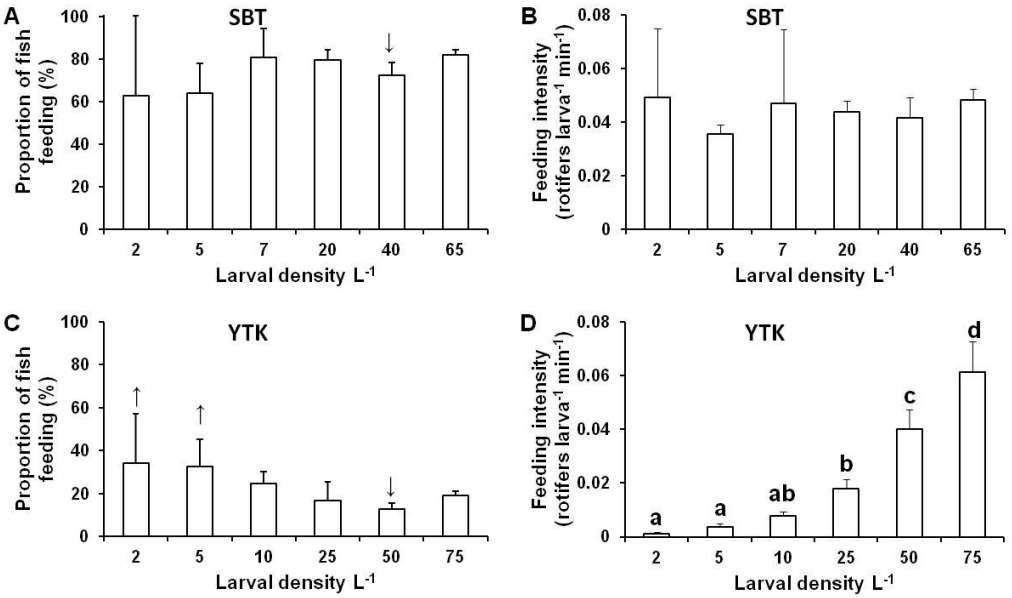

Figure 4. First-feeding response of fish larvae at different larval densities. In *Thunnus maccoyii* (SBT) (A) the proportion feeding and (B) feeding intensity, and in *Seriola lalandi* (YTK) (C) the proportion feeding and (D) feeding intensity. The arrows indicate treatments in which there were more (↑) or less (↓) larvae feeding than expected (chi-square; P ≤ 0.05). Means sharing a common letter are not significantly different. Mean + sd, n = 4.

There was no significant difference ($F_{5, 24}$ = 0.417, P = 0.831) in feeding intensity by *T. maccoyii* across the larval densities tested (0.046 ± 0.005 rotifers $larva^{-1}$ min^{-1}, Fig. 4B),whereas feeding intensity in *S. lalandi* was significantly affected by larval density ($F_{5, 24}$ = 69.551, P < 0.001) and increased incrementally from 2 to 75 larvae L^{-1} (Fig. 4D). The proportion of *T. maccoyii* feeding was twice as high as *S. lalandi*, although *S. lalandi*

had up to double the feeding intensity when exposed to a higher larval density.

Mortality, in contrast to the other two experiments (prey density and prey size), was low for *T. maccoyii* (range 0 to 17%) and *S. lalandi* (range 0 to 20%). There was no significant difference in the mortality of *T. maccoyii* among treatments (χ^2 = 2.016, df 5, P = 0.847), whereas *S. lalandi* had a higher than expected mortality at a density of 5 larvae L^{-1} (χ^2 = 12.118, df 5, P = 0.033).

2.4.2.4 Surface oil and first-feeding

The mortality of *T. maccoyii* was significantly reduced in the oil treatment (1%, mean ± sd of four aquaria) compared to the no oil treatment (17%, mean ± sd of four aquaria) (χ^2 = 12.416, df 1, P < 0.001). No difference in the proportion of fish feeding (χ^2 = 0.001, df 1, P = 0.971) or feeding intensity ($F_{5, 18}$ = 0.204, P = 0.957) was observed.

2.4.3 Mortality

Mortality recorded in prey density, and prey size and density experiments, was three times higher for *T. maccoyii* than *S. lalandi*. Mortality recorded for the larval density experiment was similar for *T. maccoyii* and *S. lalandi* (Table 2). *Thunnus maccoyii* mortalities were evident on the surface directly after transfer, and microscopic inspection at the end of each short-term feeding experiment revealed that dead larvae were in a state of decomposition, which suggested the larvae died at or soon after transfer. Surface mortality was observed in all *T. maccoyii* experiments. The high surface mortality observed in *T. maccoyii* had the potential to compromise the study if the feeding ability of the surviving larvae was affected. The short-term feeding experiment, which assessed the effect of the addition of oil on feeding and mortality, revealed oil addition significantly reduced mortality, but no difference was observed in feeding response.

2.4.4. Assessment of transfer stress from the larviculture source tank to the experimental system.

Assessment of feeding response in *T. maccoyii* was obtained from empirical evidence of three cohorts of first-feeding larvae exposed to similar conditions as described for the three *T. maccoyii* cohorts in my study. *Thunnus maccoyii* larvae were sampled from each larviculture source tank four hours after the addition of rotifers. The average proportion of *T. maccoyii* larvae feeding in the source larviculture tank was 90% with an intensity of 0.04 rotifers larva^{-1} min^{-1}, reflecting the values obtained in the experimental aquaria. In comparison, *S. lalandi* larvae were sampled from each source larviculture tank on the experimental day, four hours after the addition of rotifers. *S. lalandi* displayed a reduction in feeding in the experimental aquaria, two to four times lower, than the larviculture source tank (i.e., proportion feeding 50%, 70% and 90% with an intensity of 0.01, 0.02 and 0.03 rotifers larva^{-1} min^{-1} for the prey density, larval density and prey size experiments, respectively).

2.5 Discussion

2.5.1 Feeding response

The 4 h experiments used in my study highlighted a number of differences between *T. maccoyii* and *S. lalandi* first-feeding larvae. While both species fed in the experimental unit, *T. maccoyii* had a consistently higher proportion of larvae feeding and generally fed with greater intensity compared to *S. lalandi*. The maximum first-feeding response for *T. maccoyii* was 83% and 0.032 rotifers larva^{-1} min^{-1} compared to 23% and 0.022 rotifers larva^{-1} min^{-1} for *S. lalandi*.

First-feeding ability varies greatly among species (Houde and Schekter, 1980; Hunter, 1980). Comparing the results of first-feeding studies is complicated due to disparities between experimental conditions, which significantly affect the first-feeding response. A limited number of similar small-scale, short-duration experiments on other marine fish species confirm the relatively higher feeding ability of *T. maccoyii* over other

species. For example, striped trumpeter, *Latris lineata*, show lower first-feeding responses similar to *S. lalandi* (Cobcroft et al., 2001; Shaw, 2006), and Carton (2005) found the first-feeding response of *S. lalandi* to be comparable to the present study (proportion feeding 15 to 35% and feeding intensity 0.001 to 0.008 rotifers ingested min^{-1}).

There are a number of possible explanations for the higher feeding response of *T. maccoyii* that may be a result of visual and non-visual sensory feeding. Larger eye size improves visual ability due to an increased visual field (Job and Bellwood, 2000; Johns and Easter, 1977), however, it is unlikely that this was the cause as both species had similar relative eye sizes and the absolute eye size of *S. lalandi* was larger than *T. maccoyii*. The increased feeding response of *T. maccoyii* may be a factor of retinal morphology. Adults of tuna species possess extremely high visual acuity (Kawamura et al., 1981), which is reliant on the solid angle viewed by the eye (Fernald, 1989), the photoreceptor spacing in the retina, the proportion of cones to ganglion cells, and the focal length of the lens (Pankhurst et al., 1993). If larval *T. maccoyii* also have high visual acuity it may explain the increase in prey detection and subsequent predatory ability, although the comparative retinal morphology of early-feeding *T. maccoyii* and *S. lalandi* is currently unknown. Physical detection of prey may also be a result of mechanoreception, as the cupula of the free neuromasts slightly bend in response to prey movement (Blaxter, 1987; Kjørsvik et al., 2004; Mukai et al., 1994). Larvae with large and/or numerous free neuromasts has been shown to have increased motion detection (Mukai et al., 1994). Examination of mechanosensory development in the Pacific bluefin tuna, *Thunnus orientalis*, and the northern bluefin tuna, *Thunnus thynnus* revealed many well developed neuromasts at an early stage of development (Amoroso, 2011; Kawamura et al., 2003). This would suggest that the closely related *T. maccoyii* may also have acute mechanosensory function at an early age, contributing to increased feeding performance.

An additional theory to sensory feeding being responsible for a higher feeding response in *T. maccoyii* may be that *T. maccoyii* are faster to

learn successful feeding strategies compared to *S. lalandi*. In addition, the higher feed intake may indicate a higher metabolism and requirement for more immediate feeding by *T. maccoyii*.

The effect transfer had on the larvae revealed that while first-feeding *T. maccoyii* experienced high transfer mortality due to entrapment on the surface, the surviving larvae appeared unaffected by capture and feeding in a novel environment. In fact, feeding rates were similar in the short-term feeding experiments and larviculture source tanks, which confirms that the feeding performance of surviving *T. maccoyii* larvae was unaffected by transfer, or they recovered quickly, and that the observed feeding ability of *T. maccoyii* was relatively high. While a proportion of the *S. lalandi* cohorts consumed rotifers on 2 dph, it is well documented that first-feeding occurs on 3 dph (Carton, 2005; Chen et al., 2006; Ma and Qin, 2012). Feeding experiments were initiated on 3 dph for both species as the larvae possessed fully pigmented eyes and had fully consumed their endogenous yolk indicating the need to commence feeding, which agrees with studies reported by Woolley et al. (2009) and Chen et al. (2006). The reduced feeding response of *S. lalandi* may be attributed to greater stress levels associated with transfer than in *T. maccoyii*, a reduced need to initiate feeding and/or a reduced ability to successfully feed in a novel environment, as seen with *L. lineata* and greenback flounder, *Rhombosolea tapirina* (Cobcroft et al., 2001; Shaw, 2006). Shaw (2006) suggested that some larvae develop a search image which leads to selection for prey in a previously experienced visual environment. Transfer from a known to an unknown visual environment, where larvae have no prior experience, may disrupt the learned search pattern.

A confounding factor in the present study was that feeding experiments occurred on the first day of feeding for *T. maccoyii* (with no prior exposure to live-feed) and the second day of feeding for some *S. lalandi* larvae (with prior exposure to live-feed). Prior exposure to live prey has been shown to improve feeding performance in larvae of some marine fish (Cobcroft et al., 2001; Cox and Pankhurst, 2000; Salgado and Hoyt, 1996), however, I suggest that prior exposure did not compromise my study, as Carton

(2005) achieved similar feeding results on naïve first-feeding 3 dph *S. lalandi*. Moreover, the feeding performance of 3 dph *S. lalandi* may be expected to be higher compared to first-feeding 2 dph *S. lalandi l*arvae which would potentially make them even poorer at first-feeding compared to *T. maccoyii*.

2.5.2 Feeding experiments

2.5.2.1 Prey density and first-feeding

Thunnus maccoyii had a significant fourfold increase in the proportion of larvae feeding with increasing prey density. In contrast, prey density did not significantly affect the feeding response of *S. lalandi* although the proportion of larvae feeding did double between the lowest and highest prey density. Increasing prey density has been shown to be an important factor for feeding success in the majority of early larval feeding studies (Dou et al., 2000; Houde and Schekter, 1980; Robert et al., 2009; Seljeset et al., 2010; Shoji and Tanaka, 2004; Slembrouck et al., 2009) with studies conducted on the yellowfin tuna, *Thunnus albacores*, showing increasing survival with increasing prey density (Wexler et al., 2011).

The strong positive relationship between prey density and feeding success is thought to be a reflection of higher encounter rates between predator and prey (Shoji and Tanaka, 2004), increasing the likelihood of successful capture by larvae that are equipped with rudimentary sensory, feeding and swimming apparatus (Blaxter, 1986; Cobcroft and Pankhurst, 2003; Pankhurst and Hilder, 1998). Generally, an increase in prey density results in the feeding rate increasing until a point of satiation is reached (Houde and Schekter, 1980; Temple et al., 2004). This point was not determined in the study with *T. maccoyii*, as the feeding response was still increasing at the highest prey density offered. The results demonstrate *T. maccoyii* and *S. lalandi* have different predatory capabilities, which may have been exaggerated due to the limited time frame (4 h) available for successful feeding. Capture success at first-feeding clearly varies among species and is dependent on the ability of the larvae to detect, capture and ingest prey. *Thunnus maccoyii* are reared at higher temperatures (26 ^{0}C) compared to

S. lalandi (24 ^{0}C) and most likely have higher metabolic demands. The fact that *T. maccoyii* larvae show an increasing ability to capture rotifers with increasing prey densities, compared to *S. lalandi*, suggests that this species has greater predation capacity at first-feeding, which is most likely due to greater sensory capacity, higher requirement for feeding or less energy reserves.

2.5.2.2 Prey size, density and first-feeding

First-feeding *T. maccoyii* and *S. lalandi* larvae exposed to different combinations of prey size and prey density showed that prey size alone did not alter the feeding response. Both *T. maccoyii* and *S. lalandi* showed an increase in the proportion of larvae feeding with increasing prey density. While prey size alone had no effect on feeding rate, there was a significant increase in the number of small-strain rotifers consumed by *T. maccoyii* at the highest prey density. A possible explanation may be the ingested volume. Small rotifers had 25% of the volume of the large rotifers, requiring *T. maccoyii* to consume a greater number of rotifers in order to reach the same level of stomach fullness. Although if this was the case, it would be expected that the same response would be seen in all the small rotifer treatments. It is more likely that the high density treatment provided a sensory input that drove the feeding *T. maccoyii* larvae to continue consuming prey. While feeding can be driven by visual stimuli, it may also occur due to mechanoreception, olfaction and gustation (Blaxter, 1986). It is unlikely that the increased feeding of *T. maccoyii* in response to the smaller *B. rotundiformis* at high density, compared to the larger *B. plicatilis*, is a result of visual or mechanoreception input as it would be expected that larger rotifers would be visually more obvious and presumably produce greater movements. Olfaction and gustation stimulus, although, may explain the preference for high density small strain rotifers. Phytoplankton and zooplankton is known to exude amino acids that may induce chemotactic behaviour in larval fish (Corner and Davies, 1971; Døving et al., 1994; Olsen et al., 2004). Chromatographic analyses have shown that different zooplankton populations emit different assemblages of individual amino acids (Johannes and Webb, 1965). Feeding

experiments on turbot,*ScophthaIrnus maximus* and sole, *Solea solea,* larvae exposed to chemical stimuli revealed a species-specific attraction to chemical stimulants (Knutsen, 1992). It is possible that *B. rotundiformis*, at high density, produce a significant amount of chemoattractants that increase the attractiveness of the rotifer compared to *B. plicatilis* in *T. maccoyii.* Investigation of sensory development in *T. orientalis* revealed olfactory pits open 2 dph, well before the appearance of tastebuds at 8 (Kawamura et al., 2003). While olfactory development has not been studied for *S. lalandi*, taste bud development has been reported at 8 dph (Chen et al., 2006). As the feeding experiments in this study focused on first-feeding (3 dph), it is unlikely that the taste buds of either species played a role in feeding, although olfaction, particularly in *T. maccoyii*, may have contributed to the feeding response.

Seriola lalandi had prior exposure to large rotifers which may have biased the feeding response to favour prey that the larvae were experienced with, as has been found in other studies with different species (Cobcroft et al., 2001; Cox and Pankhurst, 2000; Salgado and Hoyt, 1996). However, this did not appear to be the case as *S. lalandi* fed equally well on small and large rotifers. Larval fish actively consume zooplankton as large as their mouth-gape will allow (Graham and Sprules, 1992). Both species ingested large rotifers at first-feeding, which indicates that their mouth gape was sufficient for this prey size and did not apparently limit prey ingestion. The consumption of larger prey increases the net rate of energy gain per predatory strike, consequently the selection of the most energetically profitable prey type has the potential to increase growth rates and decrease mortality (von Herbing et al., 2001). The use of large rotifers instead of small rotifers in the culture of *T. maccoyii* and *S. lalandi*, therefore, will maximise the energy gains of the larvae theoretically improving the larviculture of the species.

2.5.2.3 Larval density and first-feeding

The feeding response of *T. maccoyii* larvae was independent of larval density and the larvae showed neither feeding inhibition, nor feeding

advantage due to crowding from conspecifics. In contrast, there was a significant decline in the proportion of *S. lalandi* larvae feeding with increasing larval density. While my study did not investigate the effect of growth and larval density, decreased growth has been recorded at high larval densities and it has been postulated that the decline in growth may be due to stress associated with space availability (Hitzfelder et al., 2006; King et al., 2000). The stress response in fish exposed to identical conditions is species-specific in terms of magnitude, timing and duration (Fanouraki et al., 2011). Appetite reduction or the inability to initiate feeding (King et al., 2000; Wendelaar-Bonga, 1997) has also been associated with high larval densities. Deterioration of water quality associated with the metabolic outputs within the larval culture environment is thought to be a major cause of decreased growth (Baskerville-Bridges and Kling, 2000; King et al., 2000; Roo et al., 2010; Szkudlarek and Zakes, 2007). Due to the short duration of the experiment this factor is unlikely to have played a role in the current study. Despite the proportion of *S. lalandi* larvae feeding declining with larval density, feeding intensity increased significantly in those larvae that did start feeding. Contrary to reports of feeding inhibition at high larval densities, individual *S. lalandi* appeared to benefit in terms of prey capture success (Hecht et al.,1996). One explanation may be that at higher densities the larvae interfered with or distracted each other resulting in a smaller number of larvae feeding in a positive feed-back loop. The larvae that were feeding were likely to be the stronger or more capable (faster to learn and succeed at feeding behaviour) and consequently could out-compete the non-feeding larvae. Hence, once the fish had initiated feeding they continued to feed more effectively driven on by the competition or success in prey capture despite the distraction of conspecifics. Large variation in fish size has been recorded in a juvenile *S. maximus*, associated with high stocking density (Irwin et al., 1999). The authors concluded that high stocking densities of juvenile *S. maximus* suppressed the growth of some individuals and to achieve fish size homogeneity lower stocking densities were required. High larval density has been shown to improve feeding in snapper, *Pagrus auratus*, although increased larval aggression is associated with high

larval density (Hecht, et al., 1996). The observed divergence in feeding success in *S. lalandi* with increasing larval density may explain why large variations in size are often seen in commercial production of *S. lalandi* during the larval stage, with initial stocking densities ~100 larvae L^{-1}. Significant size variation in larval cohorts in *S. lalandi* has been linked to later aggression, cannibalism, and walling associated with jaw malformations (Cobcroft, et al., 2004; Cobcroft and Battaglene, 2009; Cobcroft, 2013).

2.5.3 Mortality

Mortality was three times higher for *T. maccoyii* in the prey density, and prey size and density experiments compared to *S. lalandi*. It appeared that the high mortality of *T. maccoyii* larvae was due to the larvae "sticking" on the water surface. Surface mortality (or "floating death") is common in *T. thynnus*, culture and is thought to occur when larvae are trapped by surface tension (Miyashita, 2002). Yamaoka et al. (2000) suggest that mucus excreted by larval fish may act as glue when larvae are exposed to the water surface. The relatively small size of first-feeding *T. maccoyii* larvae (3 mm) may result in an inability of the larvae to break free from the surface tension. Surface mortality of other small (≤ 3 mm) first-feeding marine larvae has often been recorded including, red spotted grouper, *Epinephelus akaara* (Yamaoka et al., 2000), Japanese flounder, *Paralichthys olivaceus* (Tagawa et al., 2004), striped bonito, *Sarda orientalis* (Kaji et al., 2003), and *T. orientalis* (Sawada et al., 2005). *Seriola lalandi* may have survived better from a combination of being larger and more capable of breaking the surface tension, being less attracted to the water surface or being less likely to possess the proposed physiological mucus response resulting in surface adhesion.

The experiment examining the effect of oil addition on larval mortality revealed that surface mortality of *T. maccoyii* larvae was significantly reduced with the addition of oil. There was no correlation between transfer mortality (observed as surface mortality), and the feeding performance of surviving larvae in the aquaria, indicating that high and variable mortality

among treatments did not affect feeding rates of *T. maccoyii*, and was unlikely to have affected the original feeding experiments.

Mortality was comparable between *T. maccoyii* and *S. lalandi* in the larval density experiment, with *T. maccoyii* experiencing a threefold reduction in mortality compared to the other two *T. maccoyii* feeding experiments. An explanation for the reduced mortality may be that the collection of higher larval numbers in a single collection beaker accumulated more oil from the larviculture source tank and reduced the surface tension in the experiment aquaria. The addition of oil has been used to reduce surface mortality in other marine species (Kaji et al., 2003; Yamaoka et al., 2000) including *T. orientalis* (Ishibashi, 2010).

2.6 Conclusion

Similarities in the early larval feeding capacities of *T. maccoyii* and *S. lalandi* might be expected based on their shared biological attributes, including their small size and fast-growing, oceanic nature. My study showed this was not the case and substantial differences exist between the species around first-feeding. The consistently higher proportion and intensity of *T. maccoyii* larvae feeding compared to *S. lalandi* clearly showed *T. maccoyii* has greater predation capacity which may be a factor of sensory ability or the need to meet higher energy requirements. High initial rotifer density (25 mL^{-1}) increased the proportion of feeding in both *T. maccoyii* and *S. lalandi* larvae. Large rotifers were a suitable sized prey for both species and ingestion of larger prey should increase energy intake. There was no effect of larval density on the feeding response of *T. maccoyii* suggesting that if water quality can be maintained, early larval rearing at densities up to and possibly above 65 larvae L^{-1} may be achievable. The situation for *S. lalandi* was different and more complicated. Increasing larval density decreased the proportion of larvae feeding but increased the intensity of feeding. The relationship between how many larvae can feed versus the average feed intake is a challenge for larviculturists and possibly explains the large variation in larval size seen at an early stage in *S. lalandi* commercial production. The small-

scale, short duration feeding experiments in my study provided useful feeding response data, although they may not be directly applicable to larval performance in a larviculture tank, and consequently experimental results should be used to select important parameters for testing in longer-term rearing trials.

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Chapter 3. The first-feeding responses of southern bluefin tuna, *Thunnus maccoyii*, and yellowtail kingfish, *Seriola lalandi*, indicate different environmental requirements in culture

3.1 Abstract

Southern bluefin tuna, *Thunnus maccoyii*, and yellowtail kingfish, *Seriola lalandi*, both have pelagic larvae, which inhabit different nutrient zones (oligotrophic and eutrophic, respectively). This study examined their visual capacity under laboratory manipulation of abiotic factors. First-feeding larvae were challenged via short-duration (4 h) feeding experiments against light intensity, turbidity, tank colour and turbulence. Visual capacity was defined by feeding performance measured as proportion and intensity of feeding. *Thunnus maccoyii* feeding performance was unaffected by light intensity (0.4 to 26.2 µmol s^{-1} m^{-2}), tank colour and turbulence (below the lethal limit). Feeding performance was significantly higher in clear water than turbid water, and increased turbidity > 25 nephelometric turbidity units reduced feeding. In contrast, *S. lalandi* did not possess the same feeding ability across a broad range of abiotic factors, indicating a narrower "environmental window" for feeding success. Increasing light intensity (0.1 µmol s^{-1} m^{-2} to 30.0 µmol s^{-1} m^{-2}) improved the proportion of larvae feeding. *Seriola lalandi* feeding intensity was significantly higher in dark blue tanks compared to green tanks and the proportion feeding higher in pink or tan tanks. Turbulence also affected feeding performance, which was significantly higher in low turbulence. Under laboratory conditions *T. maccoyii* were more efficient predators (two to three times greater), possibly reflecting a greater visual/predatory efficiency and high metabolic requirement.

3.2. Introduction

First-feeding is a critical window in the development of marine finfish larvae and is often associated with high mortality (May, 1974). The ability to detect, capture and ingest sufficient prey is crucial in making the successful transition from endogenous to exogenous feeding. The consequence of the failure to feed is mortality due to starvation (Hjort, 1914; May, 1974). Progeny of broadcast spawners typically possess limited visual ability at the time of first-feeding, with improving acuity and sensitivity concurrent with growth (Blaxter, 1986). As most fish larvae are visual feeders and require light to feed, prey detection is reliant upon the ability of the eye to collect sufficient light to form a focused image for analysis (Fernald, 1989). Consequently, the visual environment can have a significant impact on the feeding ability of first-feeding larvae (Blaxter and Jones, 1967). The marine environment has many habitats that display great optical variability. Within relatively small spatial zones, increasing depth profiles can affect a number of factors including light intensity, spectral quality, concentration of primary productivity and the speed of currents (Costello, 2009; Jerlov, 1976). Optical differences are also seen in larger spatial zones. Oligotrophic zones are characterised by the low attenuation of light due to low particulate loads whereas eutrophic zones with high particulate load exhibit rapid scatter and absorption of light (Job and Shand, 2001). Larval fish inhabit a niche within these localised environments best suited to their physiological requirements (Davis et al., 1990; Job and Bellwood, 2000).

First-feeding ability in marine fish larvae is known to vary among species with environmental variables such as light intensity, turbidity, background colour and turbulence significantly affecting the feeding response (Kimura et al., 2004; Martin-Robichaud and Peterson, 1998; Pankhurst and Hilder, 1998; Stuart and Drawbridge, 2011). Under laboratory conditions the manipulation of abiotic factors can potentially optimise visual conditions for prey detection and prey capture in larval fishes.

The southern bluefin tuna, *Thunnus maccoyii*, aquaculture industry is currently based on fattening wild-caught juveniles but is constrained by quotas (CCSBT, 2011). The first successful spawning of captive *T. maccoyii* broodstock occurred in 2008 (Thomson et al., 2010). Early-rearing trials identified significant larval mortality from first-feeding to 14 days post-hatching (dph). In comparison, while yellowtail, *Seriola quinqueradiata,* culture is also based mainly on the on-growing of wild-caught juveniles (Nakabo, 1993; Nakada, 2008), established larviculture protocols for the yellowtail kingfish, *Seriola lalandi*, have been developed in Japan, Australia and New Zealand (Benetti et al., 2005; Kolkovski and Sakakura, 2004; Poortenaar et al., 2000). However, the production of high quality *S. lalandi* juveniles is still significantly affected by poor swimbladder inflation success and high deformity rates (Kolkovski and Sakakura, 2004; Woolley et al., 2012).

Both *T. maccoyii* and *S. lalandi* larvae appear similar; they are the progeny of broadcast spawners, have oceanic planktonic larval stages, display similar larval morphology, grow quickly and are highly cannibalistic (Kolkovski and Sakakura, 2004; Thomson et al., 2010). While there is limited information available about larvae in the wild, it would appear that *T. maccoyii and S. lalandi* may occupy different ecological niches. *Thunnus maccoyii* larvae are found in oligotrophic waters of the northeastern Indian Ocean, south of Java, characterised by poor or patchy prey distribution (Davis et al., 1990; Jenkins et al., 1991; Young and Davis, 1990). While *S. lalandi* larvae have been reported to occur in surface coastal waters off New South Wales, Australia (Smith, 1987) and in near shore waters ranging to 320 km offshore, in California, USA, (Stuart and Drawbridge, 2012; Sumida et al., 1985) possibly indicating a larval life associated with prey-rich surface eutrophic conditions which are typical of coastal waters.

My study challenged *T. maccoyii* and *S. lalandi* larvae, making the transition from endogenous to exogenous feeding, to feed under varying light intensity, turbidity, background colour and turbulence treatments. Experiments were designed to expose larvae to a broad range of

conditions, within each abiotic factor, in order to determine differences in visual capacity and identify criteria which promote early-feeding success. This information is of value in highlighting areas most requiring further investigation and designing future long-term larval rearing experiments in order to improve the larviculture of both species.

3.3. Materials and methods

3.3.1 Embryo supply and rearing

Thunnus maccoyii and *S. lalandi* embryos were provided by Clean Seas Tuna Ltd, Arno Bay, South Australia. *Thunnus maccoyii* have a relatively short-spawning season, which resulted in limited embryo availability. For this reason *T. maccoyii* experiments were conducted in 2010. *Seriola lalandi* experiments were conducted in 2011. Embryos were collected from four spawning events in February 2010 for *T. maccoyii* (> 70% fertilisation) and from four spawning events in February and March 2011 for *S. lalandi* (> 80% fertilisation). Embryo rearing protocols were similar to those reported in Chapter 2 for *T. maccoyii* and *S. lalandi.* In brief, each cohort of embryos (eight in total) was reared in individual 2500 L fibreglass cylindro-conical tanks. Culture parameters and water quality followed those reported in Chapter 2, Table 1.

Thunnus maccoyii and *S. lalandi* larval rearing protocols differed slightly due to logistical and resource constraints, this included tank base colour (green or white) and turbulence source (air generated vs. water generated). Although as larvae did not have functional eyes while in these tanks until the morning of the experiment (where they were kept in relative darkness until the commencement of the experiment), it is highly unlikely that these differences affected the larvae in any way. As seen in Chapter 2, *S. lalandi* larvae were fed live prey at 2 dph in the larviculture source tank.

3.3.2 Stocking of experimental system and experimental protocol

Approximately 30 first-feeding larvae (3 dph) (Fig. 1) were siphoned from the larviculture source tank into a 250 mL collection vessel, transferred into the 3 L aquaria and then positioned in the experimental system, as described in Chapter 2. The experimental system comprised 24 individual 3 L hemispherical, plastic aquaria with black surfaces (except in the tank colour experiment). The 3 L aquaria were randomly allocated to six treatments with four replicate test aquaria, except for the turbulence feeding experiment, which consisted of four treatments and six replicates. A single fluorescent tube (NEC tri-phosphor 18 watt, FL20SSBR/ 18-HG, T8) suspended above each treatment, provided a light intensity of 30 ± 2 µmol s^{-1} m^{-2} (n = 6, mean ± standard deviation here and throughout), which was modified for the light intensity experiments. This reading was measured with a Li-Cor LI-250 light meter with an underwater flat quantum sensor LI-1925A calibrated to air. The 3 L test aquaria were left static with no aeration or water flow.

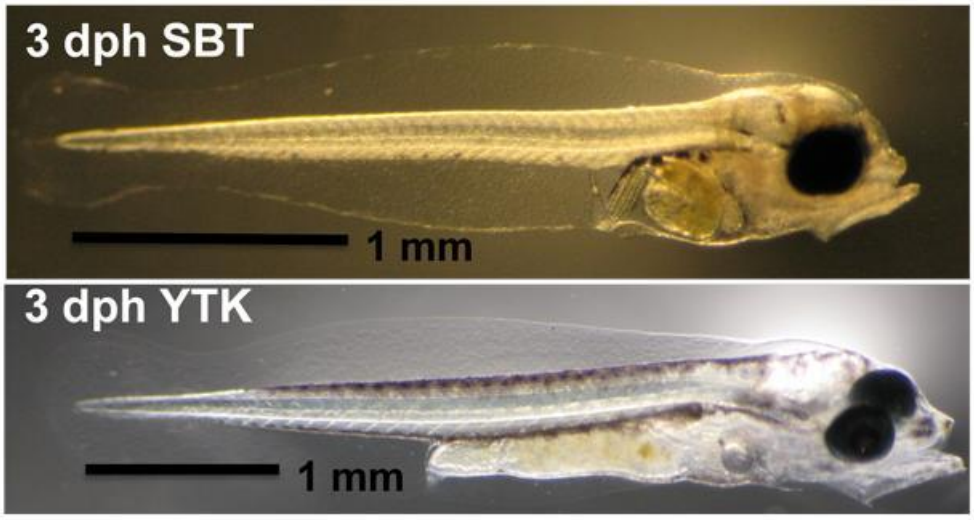

Figure 1. A photomicrograph showing first-feeding *Thunnus maccoyii* (SBT) and *Seriola lalandi* (YTK) larvae. Photos by P. Hilder.

The larvae were added to each aquarium and allowed 30 minutes to acclimate with the lights on. The experiment commenced with the addition of rotifers at a density of 2 mL^{-1} to the aquaria. Rotifers were added to one aquarium at a time with a 3 minute interval between each addition. This

allowed a 3 minute window to sample each tank at the end of the experiment, so that each tank had a precise four hour feeding period.

At the completion of the experiment, larvae were euthanised and assessed for feeding response by microscopic examination of a larval "squash". The feeding response and mortality was recorded for each treatment as the mean ± sd and determined from the following equations:

The proportion (%) of larvae feeding from the equation:

Proportion feeding = feeding larvae / live larvae x 100

Feeding intensity was expressed as rotifers larva^{-1} min^{-1} and calculated as:

Feeding intensity = rotifers ingested per feeding larva / 240 min

Where 240 min represents the experiment feeding time.

The mortality (%) was calculated from:

Mortality = dead larvae / total number of larvae x 100

On the day of the experiment, the standard lengths (taken from the tip of the upper jaw to the end of the notochord) and eye diameters (measured dorso-ventrally) were recorded for both species (n = 20). The relative eye size was determined from the following equation:

Relative eye size % = eye diameter / standard length x 100

3.3.2.1 Light intensity and first-feeding

Under clear-water conditions, *T. maccoyii* were tested at 0.0, 0.4, 1.6, 4.5, 9.9 and 26.2 µmol s^{-1} m^{-2} and *S lalandi* were tested at 0.0, 0.1, 4.0, 10.0, 30.0 and 110.0 µmol s^{-1} m^{-2}. The difference in the highest light intensity tested between the species (i.e., 26.2 µmol s^{-1} m^{-2} for *T. maccoyii* and 110.0 µmol s^{-1} m^{-2} for *S. lalandi*) was a result of the maximum light intensity achievable in the experimental system for each species in the given year. Variations in light intensity were achieved by covering aquaria with shade-cloth and/or altering the distance of the aquaria from the light

source (no change in water temperature was observed). The treatment receiving 0.0 µmol s^{-1} m^{-2} was surrounded by black plastic sheeting to eliminate all light, while the treatments receiving 26.2 µmol s^{-1} m^{-2} and 110.0 µmol s^{-1} m^{-2} were left uncovered. The addition of shade-cloth did not alter the spectral range of the light source (Cobcroft et al., 2001).

3.3.2.2 Turbidity and first-feeding

Turbidity of 0, 1, 2, 8, 25 and 50 nephelometric turbidity units (NTU) was used for *T. maccoyii* and 0, 1, 3, 9, 24 and 56 NTU for *S. lalandi*. The estimated equivalent cell density was approximately 0, 1.9 x 10^6, 3.8 x 10^6, 12.4 x 10^6, 32.2 x 10^6 and 62.5 x 10^6, respectively. The desired level of turbidity was achieved by the addition of a microalgal paste Nanno 3600®, Reed Mariculture, California (Fig. 2).

Figure 2. Photograph of turbidity levels used in turbidity and first-feeding experiments.

3.3.2.3 Tank colour and first-feeding

Six different tank colours were tested; pink, green, light blue, dark blue, tan and black (Fig. 3) (described by RGB colour separation model, Red: Blue: Green: pink (255:153:153), green (0:255:128), Light blue (0:128:255), dark blue (0:51:102) tan (255:178:102), and black (0:0:0).

Figure 3. Photograph of tank colours used in tank colour and first-feeding experiments.

3.3.2.4 Turbulence and first-feeding

Turbulence was induced by the addition of air via an airline attached to a pipette positioned at the centre of the base of the aquaria. Turbulence for *T. maccoyii* was tested at air flow levels of nil, low (19 mL min^{-1}), medium (112 mL min^{-1}) and high (396 mL min^{-1}), and nil, low (19 mL min^{-1}), medium (116 mL min^{-1}) and high (425 mL min^{-1}) for *S. lalandi*.

3.3.3 Statistics

Statistical analyses were conducted using the statistics package SPSS (SPSS statistics 19, IBM). The proportion feeding and mortality data was analysed by chi-square. Standardised residuals ≥ 2 and ≤ -2 identified significant categories in which there were more or less observed responses than expected. Intensity of feeding was analysed by one-way ANOVA. Data were evaluated for homogeneity of variance with the use of Levene's test. Tukey's post hoc test was used to describe differences between means when the ANOVA was significant. All data were presented as mean + standard deviation (four replicates per treatment unless otherwise stated). Graphs were depicted as proportion (%) to provide easy interpretation and statistical significance was accepted at $P \leq 0.05$.

3.4 Results

3.4.1 Larval standard length and size

At 3 dph, the relative eye size of *T. maccoyii* was 7.6%; the larval standard length was 3.14 ± 0.05 mm and the eye diameter was 0.240 ± 0.010 mm. While for *S. lalandi* the relative eye size was 7.5 %, the average larval standard length was 4.58 ± 0.09 mm and the eye diameter was 0.345 ± 0.003 mm. No significant difference in standard length was observed within *T. maccoyii* cohorts ($F_{4, 95} = 0.367$, $P = 0.832$) or *S. lalandi* cohorts ($F_{4, 95} = 2.371$, $P = 0.067$).

3.4.2 Feeding experiments

3.4.2.1 Light intensity

Thunnus maccoyii and *S. lalandi* were visual feeders and no feeding occurred in the dark (Fig. 4).

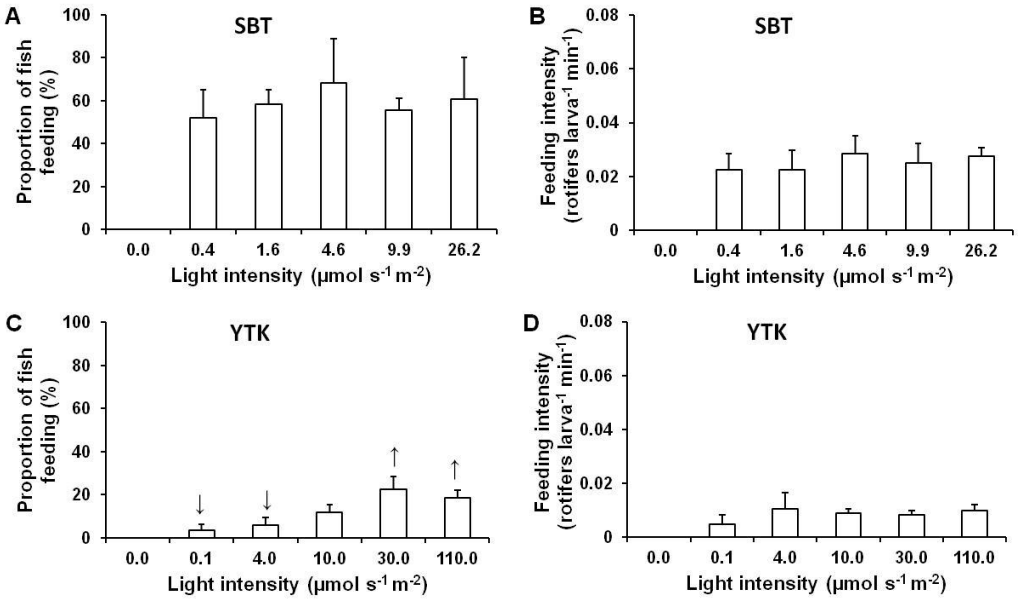

Figure 4. First-feeding response of fish larvae at different light intensities in clear-water in *Thunnus maccoyii* (SBT) (A) the proportion of larvae feeding, (B) intensity of feeding, and in *Seriola lalandi* (YTK) (C) the proportion of larvae feeding, (D) intensity of feeding. The arrows indicate treatments in which there were significantly more (↑) or less (↓) larvae feeding than expected (chi-square; $P \leq 0.05$). Mean + sd, n = 4.

The proportion of *T. maccoyii* feeding was not affected by the five light intensity levels tested (χ^2 = 2.124, df 4, P = 0.713) (Fig. 4A). In contrast, *S. lalandi* showed a significantly increased proportion of larvae feeding with increasing light intensity, where the lower light intensities had significantly less feeding responses than the higher light intensities (χ^2 = 27.234, df 4, P < 0.001) (Fig. 4C).

Feeding intensity in *T. maccoyii* and *S. lalandi* was not significantly different across the range of intensities tested ($F_{4, 15}$ = 0.768, P = 0.563 and $F_{4, 15}$ = 1.705, P = 0.201, respectively) (Figs 4B and 4D). Overall the number of *T. maccoyii* feeding was three times higher than *S. lalandi*.

3.4.2.2 Turbidity

The proportion of *T. maccoyii* larvae feeding was dependent upon turbidity level (χ^2 = 44.338, df 5**,** P < 0.001) (Fig. 5A). The highest proportion of larvae feeding occurred in the lowest turbidity treatments (0 to 2 NTU) with increasing turbidity resulting in a decreased proportion of larvae feeding.

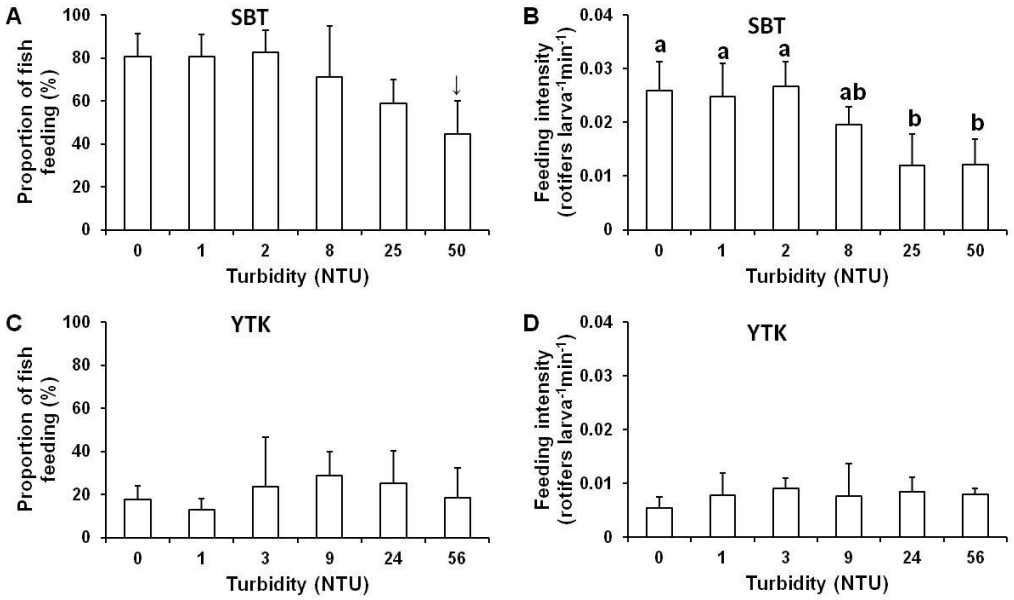

Figure 5. First-feeding response of fish larvae, with 30 ± 3 µmol s^{-1} m^{-2} light intensity, at different turbidity levels in *Thunnus maccoyii* (SBT) (A) the proportion of larvae feeding, (B) intensity of feeding, and in *Seriola lalandi,* (YTK) (C) the proportion of larvae feeding, (D) intensity of feeding. The arrow (↓) indicates treatments in which there were significantly less larvae feeding than expected (chi-square; P ≤ 0.05). Means sharing a common letter are not significantly different. Mean + sd, n = 4.

The highest turbidity level (50 NTU) had a significantly lower proportion of larvae feeding. *Seriola lalandi* showed no significant difference in the proportion of larvae feeding at any turbidity level (χ^2 = 10.966, df 5, P = 0.052) (Fig. 5C).

Feeding intensity of *T. maccoyii* (Fig. 5B) decreased as turbidity increased above 2 NTU ($F_{5, 18}$ = 6.984, P = 0.001), whereas the feeding intensity of *S. lalandi* was not significantly different at any turbidity level ($F_{5, 18}$ = 0.556, P = 0.732) (Fig. 5D). Overall the proportion of *T. maccoyii* feeding was three times higher than *S. lalandi.*

3.4.2.3 Tank colour

Tank colour had no significant effect on the proportion of *T. maccoyii* larvae feeding (χ^2 = 9.250, df 5, P = 0.100) (Fig. 6A) but had a significant effect on the proportion of *S. lalandi* larvae feeding (χ^2 = 38.877, df 5, P < 0.001) (Fig. 6C). A significantly greater proportion of *S. lalandi* larvae were observed feeding in pink and tan tanks compared to black tanks.

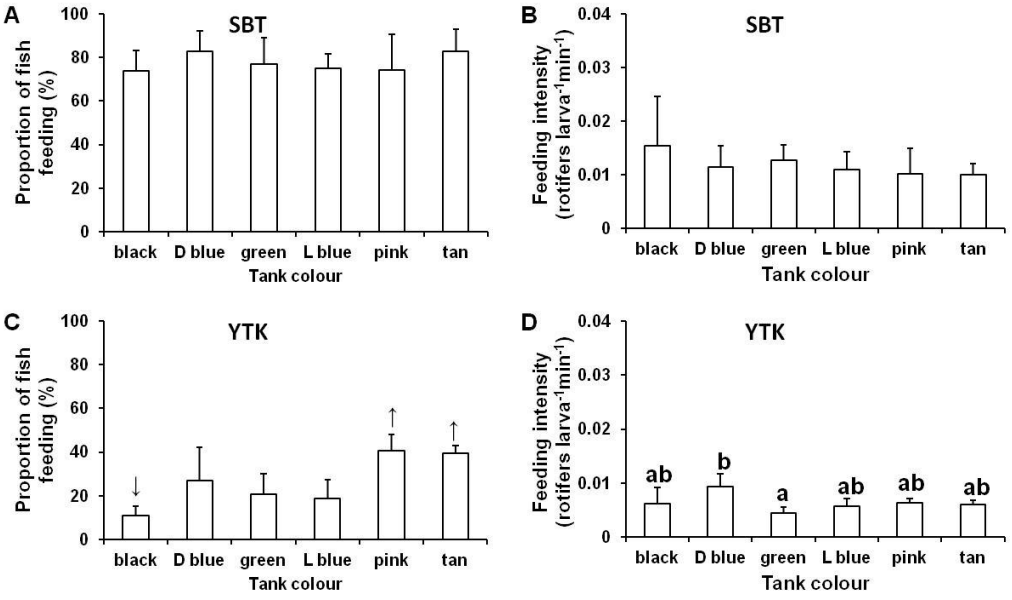

Figure 6. First-feeding response of fish larvae in six different tank colours in *Thunnus maccoyii* (SBT), (A) the proportion larvae feeding, (B) intensity of feeding , and in *Seriola lalandi* (YTK) (C) the proportion of larvae feeding, (D) intensity of feeding. The arrows indicates treatments in which there were significantly less (↓) or more (↑) larvae feeding than expected (chi-square; P ≤ 0.05). Means sharing a common letter are not significantly different. Mean + sd, n = 4.

Feeding intensity of *T. maccoyii* was unaffected by tank colour ($F_{5, 18}$ = 0.678, P = 0.645) (Fig. 6B) but was significantly different for *S. lalandi* with green tanks producing the lowest feeding intensity and dark blue tanks. producing the highest feeding intensity ($F_{5, 18}$, 3.116,P = 0.034) (Fig. 6D). In this experiment, the proportion feeding and intensity of feeding of *T. maccoyii* was approximately two times higher than *S. lalandi*.

3.4.2.4 Turbulence

Thunnus maccoyii fed in all turbulence treatments (below the lethal limit) with a similar proportion of larvae feeding at the each tested level (χ^2 = 1.632, df 2, P = 0.442) (Fig. 7A). In contrast, *S. lalandi* had a significantly higher proportion of larvae feeding in the no air or low air treatments with the highest turbulence treatment producing a significantly lower feeding response (χ^2 = 25.605, df 3, P < 0.001) (Fig. 7C).

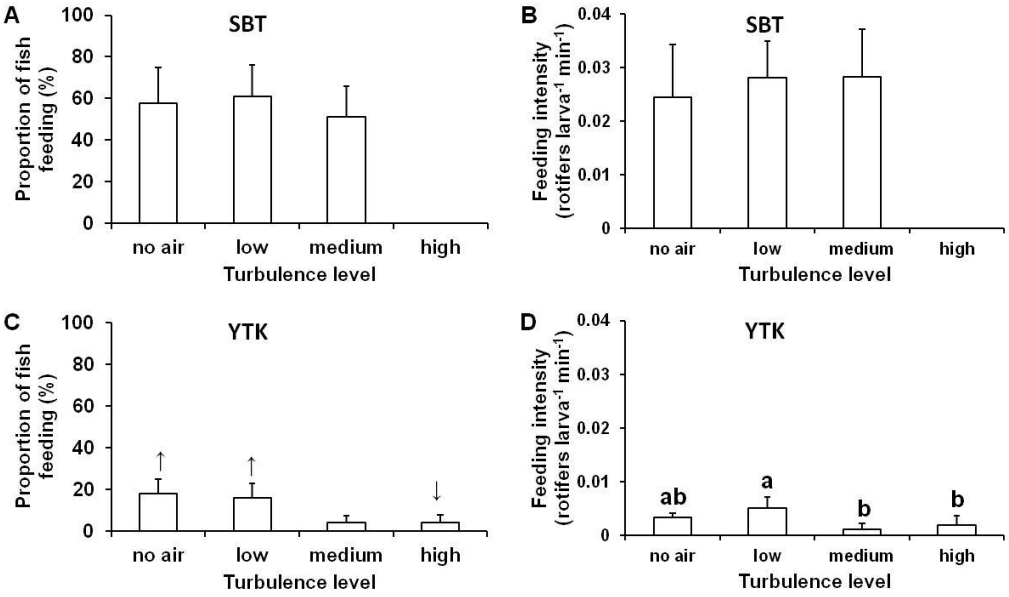

Figure 7. First-feeding response of fish larvae in different turbulence treatments in *Thunnus maccoyii* (SBT), (A) the proportion of larvae feeding, (B) intensity of feeding, and in *Seriola lalandi* (YTK) (C) the proportion of larvae feeding, (D) intensity of feeding. The arrow indicates treatments in which there were significantly less (↓) or more (↑) larvae feeding than expected (chi-square; P ≤ 0.05). Means sharing a common letter are not significantly different. Mean + sd, n = 6.

Feeding intensity was unaffected by turbulence in *T. maccoyii* ($F_{2, 7}$ = 0.160, P = 0.855) (below the lethal limit, Fig. 7B), whereas increasing turbulence (medium and high levels) significantly decreased the feeding

intensity of *S. lalandi* compared to the low turbulence level ($F_{3, 20} = 8.311$, $P = 0.001$) (Fig. 7D). Overall, the proportion feeding was approximately three times higher for *T. maccoyii* than *S. lalandi*, and feeding intensity was four and five times higher for *T. maccoyii*.

3.4.3 Larval mortality

Larval mortality was consistently higher for *T. maccoyii* across all experiments (44.0 ± 24.7%, n = 3) than *S. lalandi* (21.6 ± 12.2%, n = 4). Mortality in *T. maccoyii* could be classified as immediate (mortality observed immediately after transfer), or treatment induced (mortality due to a treatment effect). *Thunnus maccoyii* mortality was commonly observed as dead larvae on the water surface, whereas *S. lalandi* mortality was not observed on the surface and was only evident by microscopic examination at the completion of the experiment. Significant differences in mortality among treatments were observed for both *T. maccoyii* and *S. lalandi*. The highest light intensity produced significantly higher mortality in *T. maccoyii* compared to the remaining five treatment levels ($\chi^2 = 11.576$, df 5, $P = 0.041$) (53 %). In contrast, light intensity had no significant effect on mortality in *S. lalandi* ($\chi^2 = 2.802$, df 5, $P = 0.730$). Strong, air-induced turbulence significantly increased mortality for both *T. maccoyii* and *S. lalandi*. Mortality of *T. maccoyii* steadily increased from 12% at low turbulence to 83% in the highest turbulence treatment (the highest turbulence treatment was determined as the lethal limit in *T. maccoyii* and not included in analyses of feeding) ($\chi^2 = 74.921$, df 3, $P < 0.001$). In comparison, mortality in *S. lalandi* ranged between 0 and 43%, with significant mortality observed in the medium and high turbulence treatments ($\chi^2 = 32.519$, df 3, $P < 0.001$). No significant effect of mortality was observed in *T. maccoyii* or *S. lalandi* for tank colour ($\chi^2 = 4.190$, df 5, $P = 0.522$ and $\chi^2 = 3.828$, df 5, $P = 0.574$, respectively) or turbidity ($\chi^2 = 4.371$, df 5, $P = 0.497$ and $\chi^2 = 2.902$, df 5, $P = 0.715$, respectively).

3.5. Discussion

3.5.1 First-feeding experiments

My study showed that *T. maccoyii* and *S. lalandi* can feed over a broad range of abiotic factors. Both species had pigmented eyes and had consumed their yolk reserves by 3 dph, although the oil droplet was still visible, indicating the need to initiate feeding (Chen et al., 2006; Woolley et al., 2009). However, the feeding response of *S. lalandi* showed greater limitations both in terms of the proportion and intensity of feeding and the number of variables that were conducive to feeding, indicating a narrower environmental window for first-feeding success. The ability of *T. maccoyii* to feed over a broad range of abiotic conditions, in addition to a greater overall feeding success (two to three time higher) indicate a proficiency and need to actively engage quickly in first-feeding. A low first-feeding incidence in *S. lalandi* has been reported in previous studies (Carton, 2005; Stuart and Drawbridge, 2011) and the low prey density (2 rotifers mL^{-1}) selected for the feeding experiments in my study, had the potential to contribute to the low observed feeding response. However, the use of higher prey densities tested in my study (Chapter 2, 25 rotifers mL^{-1}), by Carton (2005) (6 rotifers mL^{-1}) and Stuart and Drawbridge (2011) (30 rotifers mL^{-1}) also revealed a poor feeding response supporting the argument that *S. lalandi* were not food limited in my experiment and did not show the same proficiency to commence feeding as *T. maccoyii.* Given the proven ability of *T. maccoyii* to initiate successful first-feeding, across a broad range on conditions, this would indicate that the high mortality observed during the first two weeks of commercial culture is not a failure by a high percentage of the population to initiate feeding, but more likely, attributed to environmental conditions and/or nutritional profiles of the offered prey items.

The difference in feeding ability between *T. maccoyii* and *S. lalandi* is unlikely to be due to an increased swimming ability in *T. maccoyii*, as in general, first-feeding larvae have limited locomotory ability and the smaller *T. maccoyii* would be expected to be poorer swimmers than the larger *S.*

lalandi larvae. A possible explanation for the observed difference in feeding may be adaptation of the small *T. maccoyii* for survival in a tropical oligotrophic environment typified by low or patchy prey availability. The amount of time larvae can go without food is dependent on their metabolic rate and the quantity of energy stored in the larval tissues (Fuiman, 2002). Smaller larvae have less energy reserves and a higher weight-specific metabolic energy requirement than larger larvae, and must therefore rapidly commence exogenous feeding in order to avoid starvation. A higher metabolism in *T. maccoyii* due to warmer water temperature, with increased yolk utilization, would also shorten the available “feeding window” prior to mortality due to starvation. The problem however, is that growth efficiency does not increase with higher temperatures, so larvae require increased prey consumption (Houde, 1989) which is challenging in oligotrophic waters. As such, starvation is more probable for first-feeding, small larvae in tropical waters. In contrast, *S. lalandi* larvae occur in slightly cooler water, where larger endogenous reserves may reduce the urgency to successfully initiate first-feeding. It is therefore likely that the greater predatory ability displayed by *T. maccoyii* compared to *S. lalandi* is a larval adaptation to meet their high energetic demands.

A limitation associated with short-term larval feeding experiments is transfer stress and the commencement of feeding in a novel environment. It is possible that *S. lalandi* were disadvantaged in short-term experiments because they may have required a longer acclimation period. *Thunnus maccoyii* may be more “hard-wired” to feed instantly in order to meet their energetic demands.

3.5.1.1 Light intensity and first-feeding

A major difference between the two species was the ability of *T. maccoyii* to feed equally well across all light intensities indicating greater photopic sensitivity than *S. lalandi.* The ability to feed well across a range of light intensities is uncommon among first-feeding marine finfish larvae. In general, fish larvae show increased feeding performance with increasing

light intensity (Blaxter, 1968; Carton, 2005; Cobcroft et al., 2001; Job and Bellwood, 2000; Pankhurst and Hilder, 1998). The larval eye in the majority of fish species is limited, by size constraints, to a pure cone retina (Blaxter and Staines, 1970; Cobcroft and Pankhurst, 2003; Fernald, 1989; Kotrschal et al., 1990; Margulies, 1997). This limits feeding to conditions of relatively high light intensities (photopic vision), as increased visual sensitivity to conditions of very low light (scotopic sensitivity) is associated with twin cones (Pankhurst and Butler, 1996) and rods which develop later (Kotrschal et al., 1990). In general, tuna are known to possess high visual acuity both as larvae (Margulies, 1997) and as adults (Kawamura et al., 1981; Loew et al., 2002). The advanced visual development in larval scombrids has been suggested to improve predatory performance (Margulies, 1997). Improved visual sensitivity is generally associated with larger eyes and larger retinal area for photon capture (Job and Bellwood, 2000; Johns and Easter, 1977) .The relative eye size of *T. maccoyii* and *S. lalandi* was similar, so it would appear unlikely that eye size alone was the cause of greater sensitivity. The benefit of greater visual sensitivity in first-feeding *T. maccoyii* may maximise the hours available for feeding (i.e., dawn and dusk), allow deeper vertical migration or feeding on smaller and more active prey, possibly maximising feeding opportunities while also minimising potential predation (Job and Bellwood, 2000). Photopic sensitivity observed in *T. maccoyii* has major culture implications. Benthic vertical migration is commonly observed in *T. maccoyii* (Cobcroft et al., 2012). Benthic migration may be associated with active movement away from high light intensity conditions and reduced if light intensities are lowered more than is common in other cultured marine larvae. Cultured larval tuna species are often documented to “sink” to the tank base where the subsequent exposure to high detritus and bacterial loads and hard surfaces are thought to result in major mortality (Tanaka et al., 2009). Successful culture strategies to minimise benthic migration in *T. maccoyii* larvae may include periods of low light intensity during early larval rearing. Another strategy used with oceanic larvae to minimise benthic migration has been the implementation of constant periods of light (Battaglene and Cobcroft, 2007; Partridge et al., 2011).

3.5.1.2. Turbidity and first-feeding

My results support studies that demonstrate that the effect of turbidity on feeding success is highly species-specific (Carton, 2005; Cobcroft et al., 2001; Naas et al., 1992; Stuart and Drawbridge, 2011). I showed that high turbidity (25 NTU) significantly reduced the first-feeding performance of *T. maccoyii*, although this high level of turbidity would be very uncommon in standard culture practices, whereas feeding performance of *S. lalandi* was unaffected. A similar first-feeding *S. lalandi* study by Carton (2005), while using lower turbidities (0 to 32 x 10^4 cells mL^{-1} live *Chaetoceros muelleri),* also found no significant difference in feeding performance, although significant differences were observed with increasing age. Likewise, Stuart and Drawbridge (2011), found no significant difference in 3 dph *S. lalandi* feeding response under similar light conditions to my study (1675 lx approximately equivalent to 30 µmol s^{-1} m^{-2}), although increased feeding intensity was observed under higher light intensity (14,850 lx approximately equivalent to 265 µmol s^{-1} m^{-2}) in green water compared to clear water (30 to 50 x 10^4 cells mL^{-1} algal paste, Reed Mariculture). Stuart and Drawbridge (2011) also found that while *S. lalandi* fed in both clear water and green water (0 to 5.0 x 10^5 cells mL^{-1}, algal paste, Reed Mariculture) the visual predatory ability of *S. lalandi* developed with age, so the use of green water becomes increasingly important.

The means by which turbidity improves feeding in some species is thought to occur through greater prey contrast against the background, increasing visual detection by the larvae (Miner and Stein, 1993; Naas et al., 1992; Stuart and Drawbridge, 2011; Utne-Palm, 2002). Lythgoe and Partridge (1989 and 1991) have shown that the majority of diurnal teleost fish have optical apparatus (i.e., short wavelength-absorbing cones) present in their retina that enhance contrast detection between objects and their background. Turbidity may also act by the enhancement of prey in scattering light surrounding the prey (the “halo effect”), resulting in 3-dimensional illumination of the prey (Cobcroft et al., 2001; Naas et al., 1996).

3.5.1.3 Tank colour and first-feeding

The effect of background tank colour on feeding success is species specific, due in part to a greater visual contrast of the prey against the background colour (Monk et al., 2008; Strand et al., 2007; Tamazouzt et al., 2000). While tank colour had no significant effect on the first-feeding ability of *T. maccoyii* larvae, *S. lalandi* did display significant sensitivity with increased feeding in pink and tan coloured tanks. *Seriola lalandi* have also displayed feeding preference in relation to prey colour, as Ma and Qin (2012) found brown-coloured prey significantly increased feeding intensity in the larvae compared to other coloured prey tested. As *S. lalandi* juveniles are commonly associated with floating seaweed beds where brown would be the dominant background colour (Kolkovski and Sakakura, 2004), perhaps larvae and juveniles have visual adaptations that promote feeding when animals are presented with brown backgrounds. Tank colour also affects the behaviour of light within a culture tank with darker tanks producing less reflection and back-scatter than lighter tanks (Cobcroft and Battaglene, 2009; Naas et al., 1996). It is likely that the pink and tan tanks used in my study have a high incidence of reflection and scatter possibly presenting similar conditions to wild *S. lalandi* larvae associated with eutrophic coastal waters (Smith, 1987; Sumida et al., 1985).

It would therefore appear that the increased visual detection and consumption of prey by first-feeding *S. lalandi* in pink or tan coloured tanks is likely due to a combination of background contrast and spectral sensitivity conditions, in addition to the behaviour of light in the tank environment, which appear to match those commonly associated with their wild environment. Consequently, the colour of the larviculture tank for *S. lalandi* rearing will directly affect feeding performance, where the use of pink or brown coloured tanks is likely to enable the best feeding performance, unlike *T. maccoyii* where feeding larvae are neither advantaged nor disadvantaged by the choice of tank colour.

3.5.1.4 Turbulence and first-feeding

Theoretically, increasing turbulence has the advantage of increasing the rate of predator-prey interactions (Stiansen and Sundby, 2001), provided the level of turbulence does not exceed the ability of the larvae to detect, pursue and capture prey (Kato et al., 2008). Grouper larvae ,*Epinephelus coioides*, reared in 40 L aquaria fed best at low turbulence levels 0.62 mL min^{-1} and 1.25 mL min^{-1} (Toledo et al., 2002) and studies by Shaw (2006) found turbulence levels greater than 200 mL min^{-1} significantly reduced feeding in striped trumpeter, *Latris lineata*, reared in 300 L tanks.

The difference in feeding ability observed between *T. maccoyii* and *S. lalandi* is unlikely due to increased swimming ability or speed, as suggested earlier in the discussion of feeding response, given *T. maccoyii* larvae are smaller than *S. lalandi* larvae and display proficient feeding across all turbulence levels below the lethal limit. It may be that *S. lalandi* larvae do not possess the same degree of sensory perception to allow the identification and ingestion of prey under turbulent conditions. Larval fish are equipped with a suite of senses that include vision, mechanoreception and chemoreception (Batty and Hoyt, 1995). Under turbulent conditions larvae are presented with a faster moving prey item. This challenges visual identification of the prey as well as providing greater mechanical stimulation through the movement of prey and water. While this may increase prey recognition it could also overload the sensitive neuromasts (Kawamura et al., 2003). The northern bluefin tuna, *Thunnus thynnus, and T. orientalis* have well developed mechanoreception at an early stage of larval development (Amoroso, 2011; Ghysen et al., 2012; Kawamura et al., 2003) and superficial neuromasts have been identified in the head and trunk region for *S. lalandi* that increase in number with early development (Carton, 2005). In addition, the importance of the lateral line in swimming performance in juvenile *S. lalandi* has been reported (Yanase et al., 2012). Larvae with well developed neuromasts have the potential to suffer undue stress in culture when exposed to excessive handling, aeration, vibration or turbulence (Kawamura et al., 2003). It is likely that larval *T. maccoyii* also possess well developed mechanoreception and while *T. maccoyii*

larvae experience high mortality when exposed to aeration (personal observation), high water-induced turbulence did not reduce feeding performance indicating that the mechanosensory system was not overstimulated. Larval tuna also display high visual ability at an early age which has been suggested to increase predatory ability (Margulies, 1997). Consequently, *T. maccoyii* may employ both vision and mechanoreception when feeding in turbulent waters to enhance feeding success. In comparison, *S. lalandi* had reduced feeding at the higher turbulence levels. This suggests that the speed of the prey may have been beyond their visual ability and/or the mechanoreceptor system was over stimulated. The lower proportion of first-feeding *S. lalandi* larvae feeding at higher larval densities reported in Chapter 2 may also indicate *S. lalandi* are sensitive to mechanosensory over stimulation. This has important culture implications and *S. lalandi* may do better in culture in an environment with low mechanosensory stimulation in terms of handling, vibration, con-specific densities and turbulence.

Additional studies of tuna species in the wild show wind-induced currents significantly affect feeding response (Kato et al., 2008). The authors found the effect of turbulence on larval *T. orientalis*, revealed a constant relationship between improved rotifer ingestion and increased turbulence level (up to 12 m s^{-1}) until a point where larvae could no longer feed (turbulence levels > 15 m s^{-1}). While turbulence velocity was not measured in my study, due to the limited space in the aquaria, *T. maccoyii* displayed a similar pattern to *T. orientalis* in the ability to feed well at relatively high turbulence levels. However, feeding was also proficient at low turbulence indicating a broader ability to feed across a range of turbulence levels than *T. orientalis*.

3.5.2 Mortality

Surface mortality has previously been reported in *T. thynnus* (Miyashita et al., 2000). It is commonly associated with relatively small marine fish larvae (≤ 3mm) (Kaji et al., 2003; Sawada et al., 2005; Tagawa et al., 2004; Yamaoka et al., 2000). Smaller larvae may have an inability to

break free from the surface tension and become entrapped on the surface. It is highly probable that transfer of *T. maccoyii* larvae induced significant mortality as a result of exposure of larvae to the air/water interface. In Chapter 2, surface mortality of *T. maccoyii* larvae was significantly reduced by the use of oil to break the surface tension, and feeding responses were comparable to larvae in aquaria without the addition of oil. This indicates that mortality among treatments did not affect feeding rates of *T. maccoyii*. Thus, mortality was unlikely to have affected the feeding response of larvae in the feeding experiments. In addition to transfer mortality, specific abiotic factors induced mortality in both *T. maccoyii* and *S. lalandi*. High light intensity induced significant mortality in *T. maccoyii*. This was unexpected as, in general, high light intensity is associated with a positive larval response i.e., increased feeding performance (Blaxter, 1968; Carton, 2005; Cobcroft et al., 2001; Pankhurst and Hilder, 1998; Yoseda et al., 2008). High light intensity (>2000 lx approximately equivalent to 35 µmol s^{-1} m^{-2}) has been associated with a higher incidence of surface mortality in red-spotted grouper, *Epinephelus akaara* (Setiadi et al., 2002; Yamaoka et al., 2000), and *T. orientalis* (Matsuura et al., 2010). Floating death, as it is referred to in Japan, is thought to be associated with positive phototaxis and increased exposure of larvae to the air/water interface. It appears unlikely that positive phototaxis is the cause of mortality in *T. maccoyii* due to the high photopic sensitivity displayed. Surface mortality in *T. maccoyii* may therefore not only be associated with exposure to the air/water interface but may also be associated with stress. It may be that high light intensity is a stressor to larval *T. maccoyii*. Larval fish have been reported to produce mucus under stressful conditions (Blackstock and Pickering, 1982). Yamaoka et al. (2000) suggested that mucus excreted by larval fish may act as a glue when larvae are exposed to the water surface. This agrees with finding by Kaji et al. (1995) who documented a high degree of mucous cell development in larval *E. akaara* between the ages of two and four days post hatching and found a correlation between mucous cell development and surface death.

Significant mortality was also experienced in the high turbulence treatment for *T. maccoyii* and *S. lalandi*, likely due to the direct contact of the larvae with air and/or physical damage from impact against the aquaria and/or air bubbles. Larval exposure to the water surface by upwelling currents has also been documented to cause surface mortality in the kelp grouper, *Epinephelus bruneus* (Fui Fui et al., 2012).

3.5.3 Prior exposure

Prior exposure of larvae to culture conditions has resulted in improved feeding performance in a number of marine species including *L. lineata* (Cobcroft et al., 2001), greenback flounder, *Rhombosolea tapirina* (Cox and Pankhurst, 2000), and fathead minnow, *Pimephales promelas* (Salgado and Hoyt, 1996). The exposure of 2 dph *S. lalandi* to prey the day prior to the feeding experiment had the potential to confound the experiment by falsely increasing the feeding response. This appears highly unlikely as a similar study conducted by Carton (2005) with naïve 3 dph *S. lalandi*, recorded a comparable feeding performance to my study (15 – 35% and 0.001 – 0.008 rotifers larva^{-1} min^{-1}). Prior exposure to background colour has been shown to increase the feeding performance of fish larvae (Shaw, 2006) and while my larvae were raised in green walled tanks, no feeding preference in green tanks was observed. This indicates plasticity in early larval feeding behaviour as prior experience in a specific culture environment did not constrain larval feeding.

3.6. Conclusion

The trophic environment of larval marine fish may be an indicator of the first-feeding ability of the larvae. *Thunnus maccoyii* displayed a strong feeding response to a suite of abiotic conditions highlighting a well-developed predatory capacity, possibly due to larvae in the wild being associated with oligotrophic waters. In comparison, while *S. lalandi* fed over a broad range of abiotic factors, feeding ability was restricted under a number of conditions indicating a lower predatory ability than *T. maccoyii*.

This may relate to larval life in prey rich eutrophic waters and an association with floating seaweed.

My study showed that provision of a suitable culture environment for successful feeding and survival can be achieved through the manipulation of abiotic factors and provided new information on the visual capacity of *T. maccoyii*, showing that *T. maccoyii* have a high level of photopic sensitivity at first-feeding, which is unusual among marine fish. The ability of *T. maccoyii* to feed well at low light intensity in conjunction with mortality at high light intensity would suggest that relatively low light intensities should be used at first-feeding (0.4 – 9.9 µmol s^{-1} m^{-2}). In addition, improved early-feeding in *T. maccoyii* was observed under conditions of low turbidity, with tank colour and turbulence appearing to have little effect on feeding performance. In contrast, culture requirements to maximise first-feeding in *S. lalandi* included high light intensities ≥ 30 µmol s^{-1} m^{-2}, low or no turbulence (aeration), low mechanosensory stimulation and culture in lightly coloured tanks (tan and pink). The short-term feeding experiments provided an indication of abiotic factors that affect early-feeding success and warrant further investigation in long-term, large-scale trials.

3.7 Acknowledgements

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Chapter 4. Light intensity and prey density affect the efficiency of feeding in southern bluefin tuna, *Thunnus maccoyii*, and yellowtail kingfish, *Seriola lalandi*, larvae during the rotifer-feeding phase

4.1 Abstract

I investigated the effects of light intensity and prey density on the feeding response of larval southern bluefin tuna, *Thunnus maccoyii*, and yellowtail kingfish, *Seriola lalandi*, to improve rearing conditions during rotifer feeding. The proportion of feeding and feeding intensity of *T. maccoyii* and *S. lalandi* larvae exposed to varying light intensity (0 to 100 µmol s^{-1} m^{-2}) and prey density (0.5, 1, 2, 5, 15 and 25 rotifers mL^{-1}) was tested in short-duration (4 h) experiments on 3, 6 and 9 days post hatching (dph) larvae. As light intensity increased, there was a significant improvement in feeding efficiency in *S. lalandi* at all ages. In contrast, the feeding response of *T. maccoyii* did not increase with light intensity at first feeding, and was significantly higher in older larvae at low light intensities. High light intensity induced significant mortality, associated with surface adhesion, in *T. maccoyii* but not in *S. lalandi*. Increased prey density improved feeding performance with increasing age in both species during the rotifer-feeding phase. The results show fundamental differences between the species in their light requirements, which changed over time as larval development progressed. These differences may reflect species-specific adaptations to feeding environments in the wild i.e., *T. maccoyii* larvae are associated with low or patchy prey distribution in oligotrophic waters, whereas *S. lalandi* are associated with coastal waters typified by prey-rich eutrophic conditions. The results have important implications for improving production efficiency in the hatchery.

4.2 Introduction

The progeny of broadcast spawning fish hatch in an undeveloped state and undergo rapid morphological and physiological development during their early ontogeny (Kjørsvik et al., 2004). The ability to catch sufficient prey is critical to larval survival, both in the wild and in culture, as young larvae have limited energy reserves and therefore must initiate successful feeding to avoid starvation (Hjort, 1914; May, 1974). Consequently, it is essential to optimise the early larval culture conditions to maximise feeding potential and promote larval health and survival, during the developmental phase of young larvae.

The primary sense for feeding in marine finfish larvae is vision, and light is critical to fish vision (Blaxter, 1986; Gimenez and Estevez, 2008; Kawamura et al., 1981). First-feeding larvae make the transition from endogenous to exogenous feeding equipped with only a rudimentary visual apparatus (Blaxter, 1975). As larvae develop retinal morphology changes and visual ability improves, with a subsequent improvement in prey capture success (Johns, 1982; Kotrschal et al., 1990). Historically, environmental conditions are kept constant during larval rearing, however, there is strong evidence that optimal conditions for feeding change with larval ontogeny (Carton, 2005; Cobcroft et al., 2001; Pankhurst and Hilder, 1998).

In Chapters 2 and 3, I investigated the first-feeding response of southern bluefin tuna, *Thunnus maccoyii*, and yellowtail kingfish, *Seriola lalandi*, and found that light intensity and prey density had significant effects on larval feeding and there were differences between the two species in terms of optimal conditions. Like the majority of marine fish larvae, the feeding response of *S. lalandi* improved as light intensity increased (Chapter 3) (Blaxter, 1986; Carton, 2005; Cobcroft et al., 2001; Stuart and Drawbridge, 2011; Yoseda et al., 2008). In contrast, *T. maccoyii* exhibited uniform feeding over all light intensities, indicating a high level of photopic sensitivity at first-feeding (Chapter 3). Given first-feeding *T. maccoyii* do not display the typical visual ability of marine fish larvae, as shown by *S.*

lalandi, more study was warranted to investigate the difference in the visually-mediated feeding ability of *T. maccoyii* and *S. lalandi*, and their concomitant culture requirements. Prey capture success of marine finfish at first-feeding varies among species and is dependent on the ability of the larvae to detect, capture and ingest prey (Blaxter and Staines, 1971; Houde and Schekter, 1980). While high prey densities increased the first-feeding response in *T. maccoyii* and *S. lalandi* (Chapter 2), improved visual ability with age may result in greater prey detection, thus increasing feeding at lower prey densities. Reduction in prey density would be of economic benefit and may lead to improvements in water quality and larval health through the reduction of biological and microbial load in the larval rearing system (Campbell and Buswell, 1983; Muroga et al., 1987).

The aim of this chapter was to test whether the optimal conditions for light intensity and prey density change for *T. maccoyii* and *S. lalandi* with increasing age, and to compare differences between species. The rotifer feeding stage is a critical period in larval development for *T. maccoyii* and *S. lalandi* and is often associated with high mortality, increasing the cost of seed production (Cobcroft, 2013; Cobcroft et al., 2012). My study provides a better understanding of the requirements for visually-mediated feeding efficiency of *T. maccoyii* and *S. lalandi* during their early life history, which if implemented should improve growth and survival during early culture.

4.3. Materials and methods

4.3.1 Embryo supply and rearing

Thunnus maccoyii and *S. lalandi* embryos were collected during January 2012 at the Clean Seas Tuna Ltd (CST) hatchery situated at Arno Bay, South Australia. Incubation parameters are summarised in Table 1. Egg and oil droplet diameters were recorded for each batch of embryos (n = 20).

Table 1. Incubation parameters for *Thunnus maccoyii* and *Seriola lalandi* reared in cylindro-conical fibreglass tanks. Values are mean ± sd.

Incubation parameter	*Thunnus maccoyii*	*Seriola lalandi*
Tank volume (L)	450	380
Tank colour	White	Green
Maximum stocking density (L^{-1})	200 eggs	120 eggs
Photoperiod (h L: D)	14: 10	13: 11
Light intensity (µmol s^{-1} m^{-2})	15.0 ± 2.0	0.8 ± 0.1
Temperature (^{0}C)	25.2 ± 0.6	22.6 ± 0.4
Dissolved oxygen (%)	97.5 ± 1.3	96.5 ± 0.2
pH	7.8 ± 0.1	8.1 ± 0.1
Salinity (‰)	37.2 ± 0.1	38.0 ± 0.0
Water exchange (% h^{-1})	200	20

4.3.2 Post-hatching embryo rearing and larval rearing

Thunnus maccoyii and *S. lalandi* larvae were transferred into larviculture tanks on the day of hatch (0 dph). A turbidity of 3.0 ± 1.0 nephelometric turbidity units (NTU) was achieved through the addition of an algal paste (Nanno 3600® Reed Mariculture, California). Rotifers (*Brachionus plicatilis*) enriched with Spresso® INVE, Belgium were offered twice daily from 3 dph for *T. maccoyii* and 2 dph for *S. lalandi*, at a density of 10 mL^{-1}. Larval standard length (SL) ± standard deviation (sd) and eye diameter ± sd (n = 20) were recorded for both species on 0, 3, 6, and 9 dph. Duplication of *T. maccoyii* and *S. lalandi* larval rearing systems could not be achieved due to limited access to facilities in the commercial hatchery during the spawning season.

4.3.2.1 Thunnus maccoyii

Due to logistical constraints associated with conducting research trials in a commercial facility, larval rearing for the light intensity and prey density experiments were subjected to slightly different culture regimes.

Larval rearing for the light intensity experiments was conducted in a 2,000 L white, cylindro-conical fibreglass tank. Larvae were stocked at a density of 10 ± 1 larvae L^{-1}. Water quality parameters were maintained at 25.0 ± 0.5 ^{0}C (mean ± standard deviation here and throughout), dissolved oxygen 96.0 ± 2.5%, salinity 38.0 ± 0.5 ‰ and pH 8.0 ± 0.1. Water flow

was generated by an upwelling current with an exchange rate of 18% h^{-1}. Ambient light provided a photoperiod of 14: 10 (h L: D) with an average surface light intensity of 8 µmol s^{-1} m^{-2}. Larvae used in the light intensity experiments were sourced from a single larviculture tank.

Larvae used in the prey density experiments were cultured in green-walled13, 500 L cylindrical flat-bottomed fibreglass tanks. Water quality parameters were maintained at 25.0 ± 0.5 ^{0}C, dissolved oxygen was added 108.0 ± 6.5%, salinity 37.0. ± 0.2 ‰ and pH 8.0 ± 0.1. Water flow was generated by an upwelling current with an exchange rate of 22% h^{-1}. Fluorescent, halogen lights and ambient sunlight from skylights in the hatchery, provided a photoperiod of 14: 10 (h L: D) with an average light intensity of 60 µmol s^{-1} m^{-2} at the water surface. Larvae for each short-term feeding experiment (i.e., 3, 6 and 9 dph) were sourced from three different larviculture source tanks, all in the same recirculation system.

4.3.2.2 Seriola lalandi

Larvae were reared in 380 L green-walled cylindro-conical fibreglass tanks, stocked at a density of 15 ± 1 larvae L^{-1}. Water quality parameters were maintained at 23.0 ± 1.0 ^{0}C, dissolved oxygen 91.0 ± 10%, salinity 38.0 ± 0.3 ‰ and pH 8.1 ± 0.2. Water current was generated using a single air-stone and the water exchange was 20% h^{-1}. Fluorescent tubes provided a photoperiod of 13: 11 (h L: D) with an intensity of 50 ± 3 µmol s^{-1} m^{-2} at the water surface. A single larviculture tank was designated for each experimental factor.

4.3.3 Species-specific larval rearing regimes

Larval rearing procedures differed between *T. maccoyii* and *S. lalandi* due to species-specific requirements and availability of infrastructure. Differences in water temperature and tank hydrodynamics were necessary as *T. maccoyii* required a higher culture temperature than *S. lalandi* and air-generated turbulence resulted in significant *T. maccoyii* mortality. *Thunnus maccoyii* larvae reared for the light intensity experiments were cultured in a white tank under lower light conditions. *Thunnus maccoyii*

larvae could not be cultured under high light intensity in a white tank due to significant mortality (P. Hilder, unpublished data). *Thunnus maccoyii* larvae used in the prey density experiments were reared in green culture tanks and positioned themselves in the bottom two thirds of the tank which had an equivalent maximum light intensity to the white tank (0.5 to 10.0 µmol s^{-1} m^{-2} and 6.0 to 8.0 µmol s^{-1} m^{-2}, respectively).

4.3.4 Short-term feeding experiments

Short-term feeding experiments followed protocols as reported in Chapters 2 and 3, except the number of *T. maccoyii* larvae tested on 6 and 9 dph was reduced to 20 larvae per replicate due to larval availability.

4.3.4.1 Light intensity and feeding

Experiments were conducted on 3, 6 and 9 dph for *T. maccoyii* and *S. lalandi*. *Thunnus maccoyii* were tested at light intensities of 0.0, 0.1, 1.0, 10.0, 30.0 and 100.0 µmol s^{-1} m^{-2} and *S. lalandi* were tested at 0.0, 0.1, 4.0, 10.0, 31.0 and 101.0 µmol s^{-1} m^{-2}. Light treatments were isolated with black plastic to eliminate incident light. A single fluorescent tube (NEC tri-phosphor 18 watt, FL20SSBR/ 18-HG, T8) provided light for each treatment, with varying intensities achieved through altering the distance of the aquaria from the light source and/or the addition of shade cloth over the light source. Spectral quality and water temperature were not affected by the addition of shade cloth or positioning of aquaria in relation to the light source.

4.3.4.2 Prey density and feeding

Experiments were conducted on 4, 6 and 9 dph for *T. maccoyii* and 3, 6 and 9 dph for *S. lalandi*. Prey densities were tested at 0.5, 1, 2, 5, 15 and 25 rotifers mL^{-1}.

4.3.5 Statistical analysis

Proportion feeding and mortality data were dichotomous (i.e., feeding or not feeding, and dead or alive). Consequently, the binomial data did not have a normal distribution and required chi-square analysis. Standardised

residuals ≥ or ≤ 2 denoted a significant difference (P ≤ 0.05). Graphs are presented as percentage data for easier interpretation. Feeding intensity data were analysed using one-way ANOVA. Data were evaluated for homogeneity of variance by Levene's test and Tukey's post hoc test was used to identify differences between the means when ANOVA was significant. Statistical significance was accepted at P ≤ 0.05. All statistics were carried out using the statistics package SPSS (19, IBM SPSS statistics).

4.4 Results

4.4.1 Egg morphometrics

The diameter of *T. maccoyii* and *S. lalandi* embryos was 0.95 ± 0.03 mm and 1.41 ± 0.03 mm, respectively. Oil droplet diameter of *T. maccoyii* larvae was 0.22 ± 0.01 mm and 0.30 ± 0.02 mm for *S. lalandi*. There was no significant difference in egg size within species among the batches used (*T. maccoyii* $F_{2, 57} = 3.07$, P = 0.064 and *S. lalandi* $F_{2, 57} = 1.751$, P = 0.202).

4.4.2 Larval morphometrics

Thunnus maccoyii had smaller larvae than *S. lalandi* (Table 2). Growth in larval length and eye diameter significantly increased with age (*T. maccoyii*: standard length $F_{3, 76} = 185.746$, P < 0.001, eye diameter $F_{3, 76} = 222.838$, P < 0.001; and *S. lalandi*: standard length $F_{3, 76} = 222.838$, P < 0.001, eye diameter $F_{3, 76} = 124.015$, P < 0.001).

Table 2. Standard length (SL) and eye diameter (ED) in mm at 0, 3, 6 and 9 days post-hatching (dph) *Thunnus maccoyii* and *Seriola lalandi*. Values are mean ± sd, n = 20.

	Thunnus maccoyii		*Seriola lalandi*	
Age (dph)	SL	ED	SL	ED
0	3.26 ± 0.10	0.24 ± 0.01	4.27 ± 0.11	0.30 ± 0.02
3	3.69 ± 0.07	0.25 ± 0.01	4.33 ± 0.28	0.35 ± 0.01
6	3.98 ± 0.27	0.34 ± 0.01	4.95 ± 0.25	0.41 ± 0.04
9	4.59 ± 0.21	0.41 ± 0.03	5.23 ± 0.33	0.48 ± 0.03

4.4.3 Light intensity and feeding

Thunnus maccoyii larvae did not feed in the dark and the data point was excluded from the statistical analysis. The proportion of 3 dph larvae feeding was low and unaffected by light intensity (χ^2 = 6.518, df 4, P = 0.164) (Fig. 1A), however, 6 dph and 9 dph larvae showed a significant decrease in the proportion feeding with increasing light intensity (χ^2 = 19.934, df 4, P = 0.001 and χ^2 = 10.641, df 4, P = 0.031, respectively) (Figs 1B and 1C).

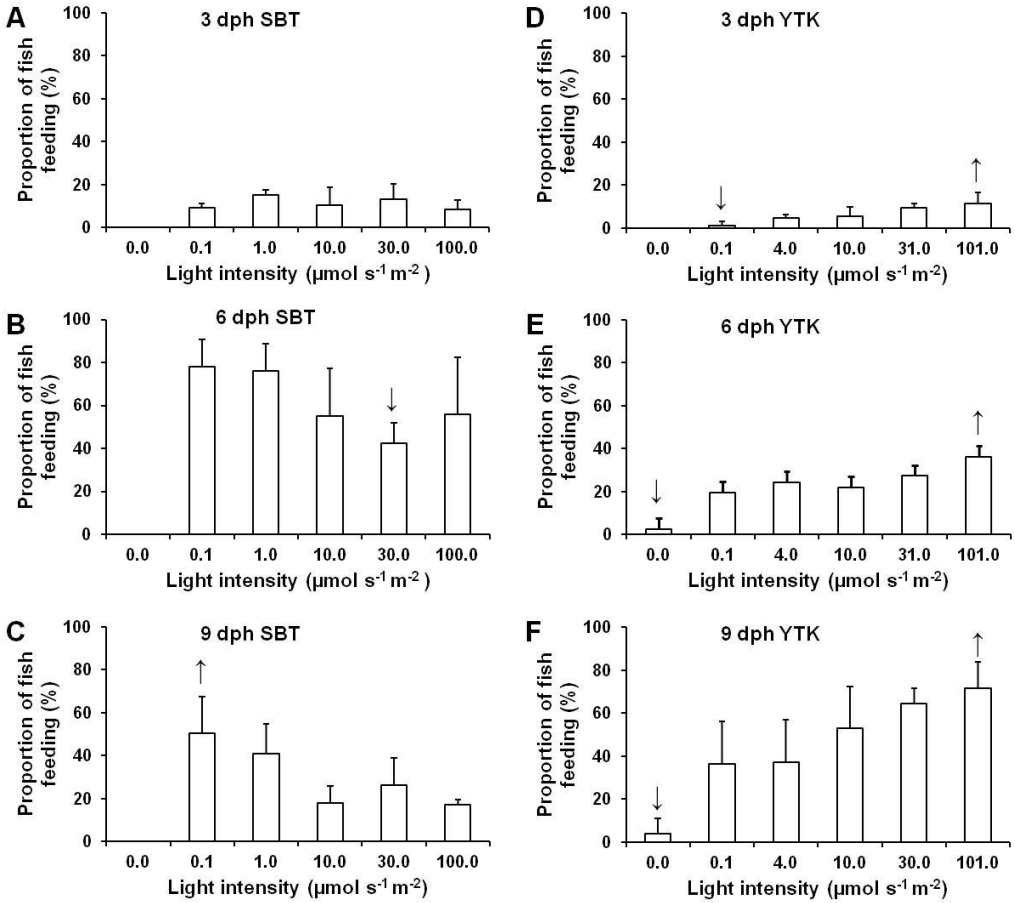

Figure 1. The proportion of larvae feeding at different light intensities in *Thunnus maccoyii* (SBT) on (A) 3 dph, (B) 6 dph, (C) 9 dph, and in *Seriola lalandi* (YTK) on (D) 3 dph, (E) 6 dph, and (F) 9 dph. The arrows indicate treatments in which there were significantly more (↑) or less (↓) larvae feeding than expected (chi-square; P ≤ 0.05). Mean + sd, n = 4.

The overall proportion of *T. maccoyii* larvae feeding increased between 3 and 6 dph from 15% to 78% and then declined to 50% on 9 dph. *Seriola lalandi* did not feed in the dark on 3 dph, although a small amount of feeding was observed on 6 dph and 9 dph. The proportion of *S. lalandi*

larvae feeding at all ages significantly increased with increasing light intensity (3 dph, χ^2 = 11.193, df 4, P = 0.024, 6 dph, χ^2 = 28.410, df 5, P < 0.001 and 9 dph, χ^2 = 34.908, df 5, P < 0.001) (Figs 1D-1F). The proportion of *S. lalandi* feeding was generally lower than *T. maccoyii* up to 6 dph, and increased with age from 11% at 3 dph to 71% at 9 dph.

Increasing light intensity had no significant effect on the feeding intensity of *T. maccoyii* at 3 dph ($F_{4, 15}$ = 0.158, P = 0.956) and 9 dph ($F_{4, 15}$ = 1.504, P = 0.251) (Figs 2A and 2C).

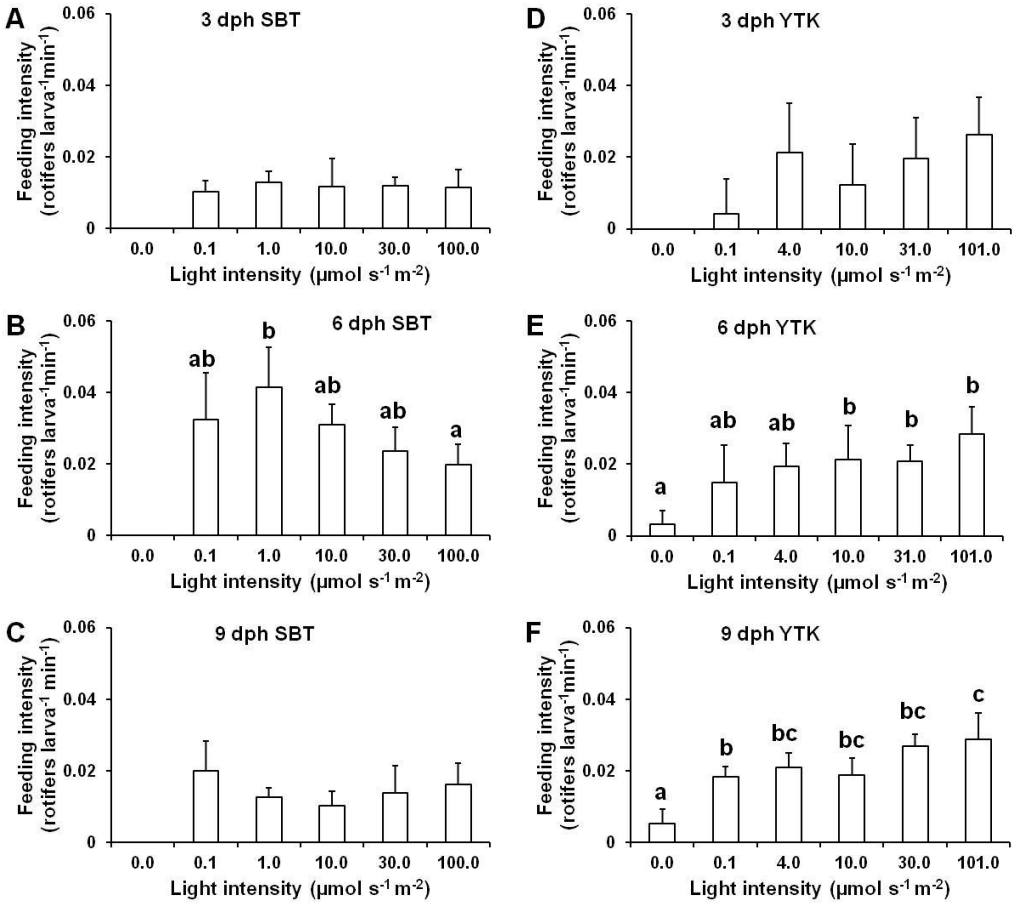

Figure 2. The feeding intensity of larvae fed at different light intensities in *Thunnus maccoyii* (SBT) on (A) 3 dph, (B) 6 dph, (C) 9 dph, and in *Seriola lalandi* (YTK) on (D) 3 dph, (E) 6 dph, (F) 9 dph. Means sharing a common letter are not significantly different. Mean + sd, n = 4.

There was significantly higher feeding intensity at 1.0 than at 100.0 µmol s^{-1} m^{-2} in 6 dph larvae ($F_{4, 15}$ = 3.529, P > 0.001) (Fig. 2B). There was no significant change in *S. lalandi* feeding intensity on 3 dph ($F_{4, 15}$ = 0.917, P = 0.160), although a trend towards increasing feeding intensity with increasing light (Fig. 2D) was observed, and a significant increase in the

same direction occurred in both 6 and 9 dph larvae ($F_{5, 18} = 5.103$, P = 0.004 and $F_{5, 18} = 13.099$, P > 0.001, respectively) (Figs 2E and 2F).

Thunnus maccoyii mortality ranged from 13% to 51% and was consistently higher than *S. lalandi* mortality which ranged from 2% to 17% and which was not significantly different among the treatments with age (3 dph, χ^2 = 0.835, df 5, P = 0.975, 6 dph, χ^2 = 1.189, df 5, P = 0.946 and 9 dph, χ^2 = 4.800, df 5,P = 0.441 (Figs 3D-3F).

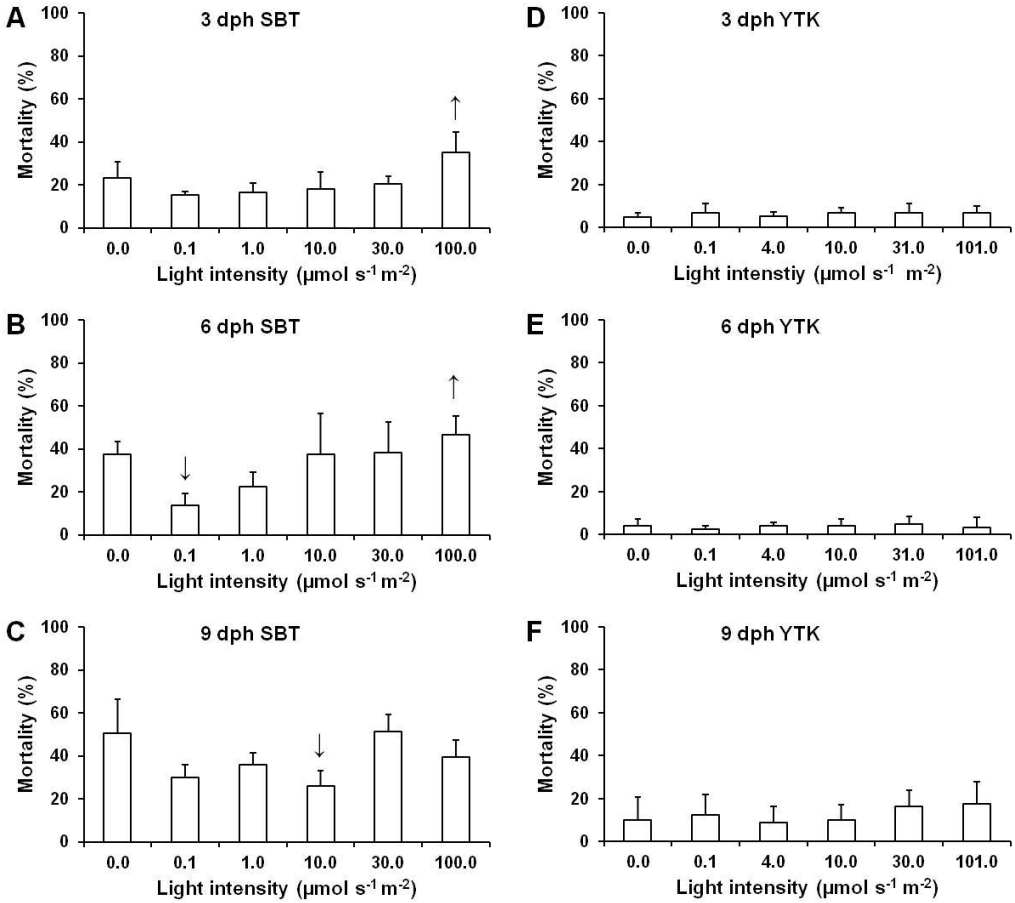

Figure 3. The mortality of larvae at different light intensities in *Thunnus maccoyii* (SBT) on (A) 3 dph, (B) 6 dph, (C) 9 dph, and in *Seriola lalandi* (YTK) on (D) 3 dph, (E) 6 dph, (F) 9 dph. The arrows indicate treatments in which there was significantly more (↑) or less (↓) mortality than expected (chi-square; P ≤ 0.05). Mean + sd, n = 4.

Mortality of *T. maccoyii* significantly increased with higher light intensity in 3 dph larvae (χ^2 = 18.706, df 5, P = 0.002) and 6 dph larvae (χ^2 = 26.074, df 5, P > 0.001) with the greatest mortality occurring in the highest light intensity treatment (Figs 3A and 3B). By 9 dph, chi-square analysis showed mortality was no longer induced by high light intensity (Fig. 3C),

instead better than expected survival was observed in the 10 µmol s^{-1} m^{-2} treatment (χ^2 = 16.735, df 5, P = 0.005).

4.4.4 Prey density and feeding

Increasing prey density significantly increased the proportion of *T. maccoyii* feeding across all ages tested (3 dph, χ^2 = 38.279, df 5, P > 0.001, 6 dph, χ^2 = 33.719, df 5, P > 0.001 and 9 dph, χ^2 = 36.914, df 5, P > 0.001, respectively) (Figs 4A-4C).

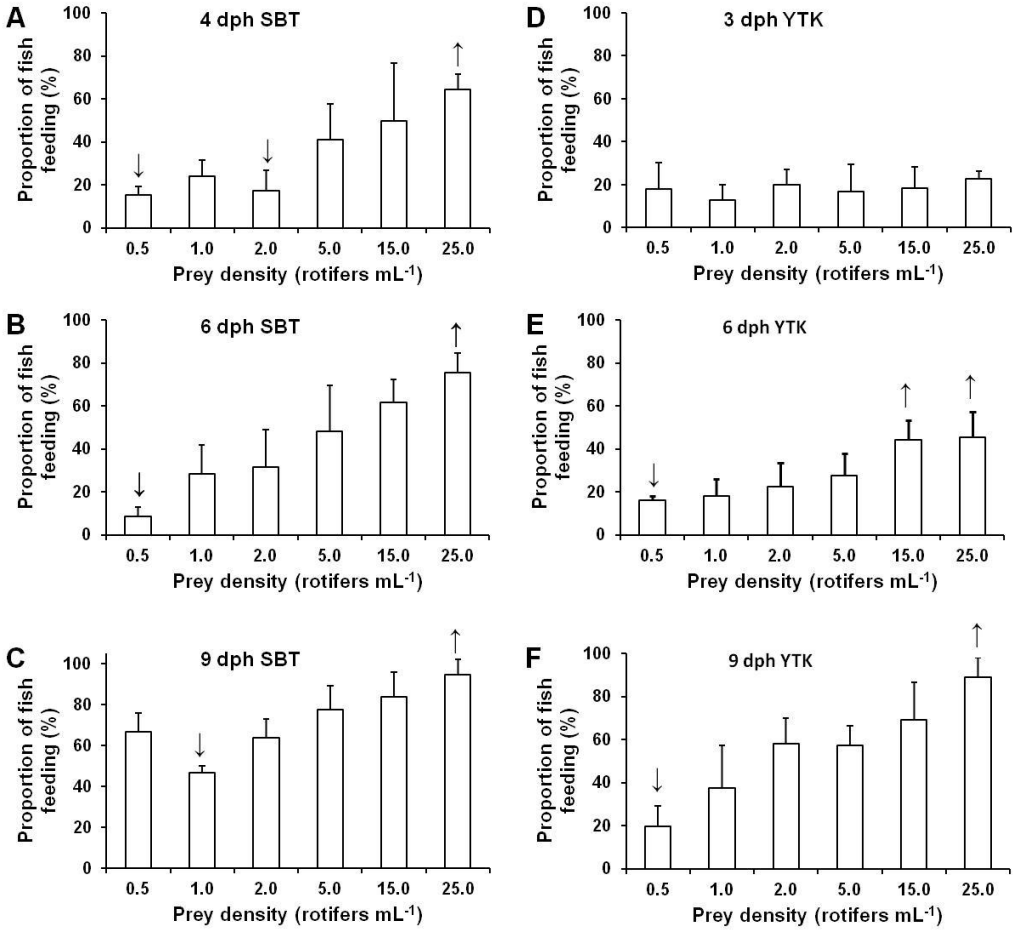

Figure 4. The proportion of larvae feeding at different prey densities in *Thunnus maccoyii* (SBT) on (A) 4 dph, (B) 6 dph, (C) 9 dph, and in *Seriola lalandi* (YTK) on (D) 3 dph, (E) 6 dph, (F) 9 dph. The arrows indicate treatments in which there were significantly more (↑) or less (↓) larvae feeding than expected (chi-square; P ≤ 0.05). Mean + sd, n = 4.

While 3 dph *S. lalandi* showed no significant relationship between prey density and the proportion of larvae feeding (χ^2 = 3.056, df 5, P = 0.691), 6 dph and 9 dph larvae had a significant increase in the proportion of larvae

feeding with increasing prey density (χ^2 = 23.641, df 5, P > 0.001 and χ^2 = 42.350, df 5, P > 0.001, respectively) (Figs 4D-4F).

Feeding intensity was not affected by prey density for either 4 dph *T. maccoyii* ($F_{5, 18}$ = 0.982, P = 0.455) (Fig. 5A) or 3 dph and 6 dph *S. lalandi* larvae ($F_{5, 18}$ = 0.656, P = 0.691 and $F_{5, 18}$ = 0.956, P = 0.470, respectively) (Figs 5D and 5E). At 6 dph and 9 dph, *T. maccoyii* larvae significantly increased feeding intensity with increasing prey density ($F_{5, 18}$ = 2.852, P = 0.046 and $F_{5\ 18}$ = 5.746, P = 0.003, respectively) (Figs 5B and 5C), with a similar effect for 9 dph *S. lalandi* larvae ($F_{5, 18}$ = 3.233, P = 0.030), although Tukeys post-hoc test did not distinguish significantly different groups (Fig. 5F).

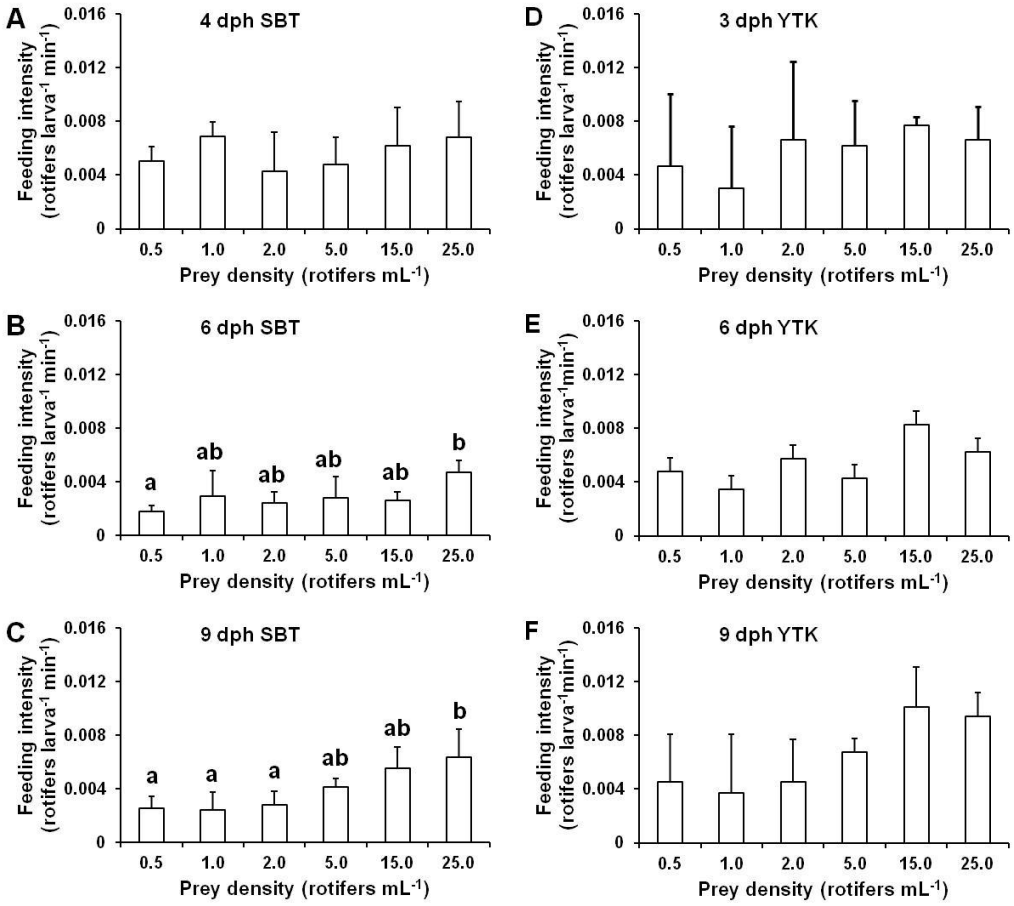

Figure 5. The feeding intensity of larvae fed at different prey densities in *Thunnus maccoyii* (SBT) on (A) 4 dph, (B) 6 dph, (C) 9 dph, and in *Seriola lalandi* (YTK) on (D) 3 dph, (E) 6 dph, (F) 9 dph. Means sharing a common letter are not significantly different. Mean + sd, n = 4.

Prey density had no significant effect on larval mortality for either *T. maccoyii* (4 dph, χ^2 = 10.381, df 5, P = 0.065, 6 dph, χ^2 = 7.338, df 5, P =

0.197 and 9 dph, χ^2 = 0.875, df 5, P = 0.972) or *S. lalandi* (3 dph χ^2 = 7.518, df 5, P = 0.185 and 6 dph χ^2 = 3.248, df 5, P = 0.662). Analysis of 9 dph *S. lalandi* mortality data failed the assumptions of chi-square as 50% of the frequency categories had expected counts less than five; however mortality was low ranging between 1% and 5 %. Overall mortality for both species was low, ranging from 3% to 14% for *T. maccoyii* and from 1% to 10% for *S. lalandi*. The acquisition of *T. maccoyii* larvae for the prey density experiments from three separate tanks did not confound the study as larval performance, determined from feeding, mortality and growth patterns in the three larviculture source tanks, were similar.

4.5 Discussion

4.5.1 Light intensity

The results clearly demonstrate changes in feeding performance of both species with age. The different feeding response of *T. maccoyii* and *S. lalandi* to light intensity highlights a fundamental difference between the species, which increases in magnitude with age. First-feeding *S. lalandi* larvae, like the majority of marine finfish larvae, exhibit improved feeding performance as light intensity increases (Cobcroft et al., 2001; Downing and Litvak, 2001; Pankhurst and Hilder, 1998; Stuart and Drawbridge, 2011). This is most likely explained by the eyes of first-feeding larval fish consisting of a pure cone retina (simplex retina), that restricts feeding to conditions of relatively high light intensity (Blaxter and Staines, 1970; Kotrschal et al., 1990). The improved feeding of *S. lalandi* larvae, in response to higher light intensities, may represent larval adaptations to natural conditions as wild *S. lalandi* larvae are associated with surface coastal waters (Smith, 1987; Sumida et al., 1985) where light intensity is generally higher.

The overall proportion of *S. lalandi* larvae feeding and the intensity of feeding improved with age. It is likely that the increased feeding ability of *S. lalandi* with age was associated with increasing eye size, cone diameters, visual acuity, and improved locomotory ability associated with

developing musculature and fins. The capacity of first-feeding *T. maccoyii* larvae to feed equally well across a broad range of light intensities at first-feeding, and then display increasing photopic sensitivity with larval development, indicates rapid morphological development of their visual system. This suggests that *T. maccoyii* larvae possess specific visual adaptations for feeding in lower light intensities which differs fundamentally to *S. lalandi*.

The importance of chemoreception and mechanoreception in the feeding response of *T. maccoyii* and *S. lalandi* has been discussed in Chapters 2 and 3. The apparent overstimulation of the mechanoreceptors in high turbulence treatments in *S. lalandi*, and olfactory preference for small strain rotifers at high density by *T. maccoyii*, suggests the importance of the non-visual senses for feeding in both species. No feeding was observed in the dark for *T. maccoyii*, and *S. lalandi* displayed a small amount of feeding on 6 and 9 dph, suggesting both species are visual feeders and that the sole use of the other senses (mechanoreception and chemoreception) is unlikely. However, the other senses may be used in conjunction with vision, particularly in *S. lalandi* where feeding in the dark was observed. Carton (2005) also reported non-visual feeding in *S. lalandi* larvae (3 to 7 dph) and suggested feeding was most likely due to mechanoreception. Taste bud development in *S. lalandi* does not occur until 8 dph, so it is highly likely that mechanoreception plays a major role in early non-visual feeding, although chemoreception due to olfaction may also contribute (Chen et al., 2006).

While the visual mechanics of photopic sensitivity in *T. maccoyii* were previously unknown, the visual sensitivity shown by *T. maccoyii* indicates that they are capable of efficiently feeding in very low light intensity. This would increase the feeding opportunities when light intensity is low, i.e., at dawn and dusk and also in deeper water. Vertical stratification of larval fish species has been well documented (Boehlert and Mundy, 1994; Davis et al., 1990). The limit of vertical migration is determined by the depth at which the minimum light intensity available allows prey capture (Job and

Bellwood, 2000). Feeding at depth has the potential benefit of maximising feeding on zooplankton which also changes their distribution, being generally deeper during the day and feeding in surface waters at night (Gerking, 1994). A further benefit would be to minimise predation by animals with higher visual feeding thresholds (Job and Bellwood, 2000). Studies by Young and Davis (1990) on wild tuna larvae reported spatial separation in the water column of *T. maccoyii*, albacore, *Thunnus alalunga,* and skipjack tuna, *Katsuwonus pelamis*. *Thunnus alalunga* were generally associated with surface waters (0 to 2 m), while only a small number of *T. maccoyii* and no *K. pelamis* were found in the surface waters. The authors suggest the differences observed in their diets reflect separation in their feeding habits, which has been shown to be an adaptation to conditions of low prey availability (Govoni et al., 1983).

Ontogenetic changes in feeding behaviour observed in *T. maccoyii* and *S. lalandi* with age have important culture implications. If *T. maccoyii* are visually adapted to feed under low light and *S. lalandi* are visually adapted for feeding under high light, different culture parameters are required to reflect these needs, and may explain previously reported aberrant behaviours and development in both species (Cobcroft, 2013; Cobcroft et al., 2012; Hutchinson, 2009; Woolley et al., 2012a). Sinking mortality commonly described in *T. maccoyii* may be a physiological response to avoid high light and my study has shown feeding will be significantly reduced at higher light intensity.

In *S. lalandi* larvae, low light intensity significantly reduces growth, survival and swimbladder inflation (Cobcroft, 2013; Stuart and Drawbridge, 2011; Woolley et al., 2012). Swimbladder inflation is affected by a number of abiotic and biotic factors including light intensity, salinity, temperature and water currents (Battaglene et al., 1994; Divanach et al., 1997; Johnson and Katavic, 1984; Saillant et al., 2003; Trotter et al., 2003), although genetic influence, both paternal and maternal, has been shown to also affect inflation (Peruzzi et al., 2007). High light intensity has been proven to significantly increase swimbladder inflation in *S. lalandi*, with the greatest swimbladder inflation rates observed at the highest light intensity

tested in a number of studies, e.g., 590 µmol s^{-1} m^{-2} (Woolley et al., 2012) and 275 µmol s^{-1} m^{-2} (Stuart and Drawbridge, 2011). While high light intensity has been associated with swimbladder hyperinflation in sea bass larvae, *Dicentrarchus labrax* (Johnson and Katavic, 1984), no hyperinflation was reported in the present study. Lack of swimbladder inflation, or hyperinflation in larval fish has major culture implications including higher mortality (Trotter et al., 2003), malformation (Chatain, 1994; Cobcroft, 2013), increased energetic requirements (Marty et al., 1995) and slower growth (Battaglene and Talbot, 1992). Definitive factors that affect swimbladder inflation in *T. maccoyii* have not been identified (Woolley et al., 2013). Light intensity (24 and 49 µmol s^{-1} m^{-2}) does not appear to have a significant effect on swimbladder inflation (Cobcroft et al., 2012). In the closely related Pacific bluefin tuna, *Thunnus orientalis*, initial swimbladder inflation could be improved if oil film removal occurred at 3 dph and if this window was missed by a day poorer swimbladder inflation occurred (Kurata et al., 2012).

In my study swimbladder inflation rates for the larvae in the larviculture source tanks were first recorded at 4 dph for *T. maccoyii* and 3 dph for *S. lalandi*. Woolley et al. (2013) reported swimbladder inflation for *T. maccoyii* in the evening 3 dph and for *S. lalandi* on 3 dph. My study did not investigate the relationship between initial swimbladder inflation and abiotic factors because the duration of the experiments was short. It is important to note the feeding experiments conducted on 3 dph generally occurred prior to swimbladder inflation and optimal conditions for feeding may not be the same for swimbladder inflation. As feeding experiments at 6 dph and 9 dph generally occur post swimbladder inflation the recommendations for abiotic factors would not be confounded by factors impacting on swimbladder inflation. Manipulation of light intensity to provide optimum rearing conditions in *T. maccoyii* and *S. lalandi* is of critical importance in the production of quality seed stock.

In addition to the feeding behaviour of larvae in response to light intensity, the mortality experienced was also significantly different between the two species. While mortality in *S. lalandi* larvae was unaffected by light

intensity, the effect on *T. maccoyii* was rapid and significant. *Thunnus maccoyii* mortalities in high light intensity, presented as dead floating larvae, which was consistent with findings from previous short-term first-feeding experiments (Chapter 3). Cultured tuna species commonly experience high mortality, particularly during early larval rearing (Davis et al., 1991; Fukuda et al., 2010; Ishibashi et al., 2009; Kaji et al., 1996; Margulies, 1997; Sawada et al., 2005). Matsuura et al. (2010) suggest that the primary problem in the production of Pacific bluefin tuna, *Thunnus orientalis* is related to their vision and may explain the floating, sinking and collision deaths. *Thunnus maccoyii* light-induced mortality was more apparent during the early life history (i.e., 3 dph and 6 dph) with no significant mortality evident in 9 dph larvae. A possible explanation for the change in the observed mortality with increasing age is given by Kaji et al. (1995). The authors hypothesised that there is a strong correlation between surface death and mucous cell development in red-spotted grouper, *Ephinephelus akaara*, larvae. Mucus production induced by stress or physical factors is thought to act as an adhesive ”sticking” larvae to surface waters (Blackstock and Pickering, 1982; Kaji et al., 1995; Yamaoka et al., 2000). Surface mortality in *T. maccoyii* larvae may be mitigated at 9 dph if mucous cell development diminishes during the early larval history as observed in the *E. akaara* (Kaji et al., 1995) or it may be a result of increased swimming ability and or body density of the larger larvae (Woolley et al., 2013). Although significant mortality was not observed in 9 dph larvae, it appeared that high light intensity was still a significant stressor, as the proportion of 9 dph larvae feeding was significantly less at higher light intensities. Identification of decreased feeding and high mortality in *T. maccoyii* larvae exposed to high light intensity, during the rotifer feeding period indicates that improved culture of the species may be achieved through the use of lower light conditions.

The high surface mortality observed in *T. maccoyii* may also be associated with swimbladder inflation. Mueller & Neuhauss (2010) discuss how larval fish, with simple nervous systems, are required to detect and react to stimuli, and for visual animals the attraction or aversion to light is

of paramount importance. In general, fish larvae exhibit positive phototaxis in response to light (Ishida, 1987 cited in Kawamura et al., 2003). Surface mortality in *T. maccoyii* may be associated with positive phototactic behaviour and attempts by larvae to gulp air from the surface for swimbladder inflation increasing the exposure of the larvae to the air/surface interface resulting in mortality.

4.5.2 Prey density

Thunnus maccoyii and *S. lalandi* larvae, like many marine fish larvae, exhibit the same relationship between increasing prey density and improved feeding (Dou et al., 2000; Houde and Schekter, 1980; Robert et al., 2009; Seljeset et al., 2010; Shoji and Tanaka, 2004; Slembrouck et al., 2009), whereby higher prey density increases the likelihood of prey encounter by larval fish and ensuing feeding success (Houde and Schekter, 1980). With increasing age, the predatory ability of *T. maccoyii*, at low prey densities, increased potentially towards feeding being independent of prey density, while throughout the experimental period *S. lalandi* required high prey density to maximise feeding success. This indicated that *S. lalandi* do not show the same ability as *T. maccoyii* in the detection and ingestion of prey and it is likely that with increasing age, lower prey densities in long-term rearing trials would not restrict *T. maccoyii* larval feeding, whereas low prey density may restrict *S. lalandi* feeding. A number of species exhibit initial density-dependent feeding, although with increasing age and larval development feeding shifts to density-independent feeding as seen in *T. maccoyii* (Dou et al., 2000; Shaw, 2006). A possible explanation in the shift from density-dependent to density-independent feeding may be that with increased larval development the visual capacity of the larvae improves. Consequently, the visual field of the larvae contains a greater number of prey items increasing the detection and potential capture of prey (Shaw, 2006). Houde and Schekter's (1980) investigation of prey capture success in relation to prey density and development in three marine larval fishes (bay anchovy, *Anchoa mitchilli*, sea bream, *Archosargus rhomboidalis,* and lined sole, *Achirus lineatus*), also revealed different feeding strategies in

the utilisation of available prey sources. While *A. rhomboidalis* displayed better growth under normal prey conditions, *A. mitchilli* exhibited opportunistic behavior with greater predatory ability at higher prey density increasing prey consumption. In comparison, *A. lineatus* had the poorest predatory ability, however, exhibited flexibility in prey choice during low prey density conditions. The authors concluded that while the early life history of larval fish in the wild is probably under the control of prey availability, species-specific feeding strategies are an important determinant in recruitment success. It appears that *T. maccoyii* and *S. lalandi* also exhibit species-specific feeding strategies. The proficient feeding of *T. maccoyii* may be a larval adaptation to survival in tropical oligotrophic waters where rapid yolk utilization at first-feeding and low or patchy prey availability increases the likelihood of starvation (Jenkins et al., 1991; Rochford, 1962). A strong relationship between prey density and feeding success has been reported in wild *T. maccoyii* larvae supporting this theory (Young and Davis, 1990). In contrast, the larger first-feeding *S. lalandi* larvae, which possess a greater endogenous reserve and develop in tropical-temperate eutrophic waters, do not display the same larval predatory ability (Chapters 2 and 3). The consumption of prey has been linked to mortality in cultured larval *S. lalandi*, as satiated larvae exhibit increased larval body density prior to dusk which is thought to lead to the larvae sinking at night and subsequent mortality (Woolley et al., 2012b). The authors evaluated larval body density to determine the latest time in the day feed could be added and still maintain larval neutral buoyancy at dusk, which significantly reduced larval mortality.

As feeding in larval fish is a learned behaviour, prior feeding experience has the potential to bias first-feeding experiments (Cobcroft et al., 2001; Cox and Pankhurst, 2000; Salgado and Hoyt, 1996; Shaw, 2006). The experiment conducted on 4 dph *T. maccoyii* had the potential to confound the study as experimental animals had prior feeding experience. The results from comparable short-term feeding experiment evaluating *T. maccoyii* feeding response and prey density reported equivalent feeding responses both in terms of the proportion and intensity of *T. maccoyii*

feeding (Chapter 2) to the current study, suggesting that the results are not confounded.

4.5.3 Application of short-term feeding experiments

First-feeding results in the present experiment correlate closely with previous studies investigating the first-feeding response of *T. maccoyii* and *S. lalandi* to abiotic and biotic factors (Chapters 2 and 3). While short-term feeding experiments are only a snap-shot of the feeding experience and have limitations (transfer stress, transfer mortality and surface effects due to vessel size), they do allow the rapid collection of well replicated data that identifies species-specific responses to culture variables that warrant further long-term investigation.

4.6 Conclusion

My study identified critical differences in the culture requirements of *T. maccoyii* and *S. lalandi* larvae. The divergent response to light intensity, both in terms of feeding and mortality, revealed the necessity for low light conditions for the photopically sensitive *T. maccoyii* larvae and high light conditions for *S. lalandi* larvae. These results suggest that culture of larval *T. maccoyii* at lower light intensity should increase survival and growth by improved feeding performance, while also reducing surface and sinking mortality. In contrast, optimal feeding performance in *S. lalandi* larvae will most likely be achieved at a relatively higher light intensity. While increasing prey density significantly increased prey intake, the greater detection and consumption of prey by *T. maccoyii* at lower densities indicates the potential to reduce prey densities more rapidly in culture than for *S. lalandi*. This would allow a reduction in the biological load of the culture system and potentially improve culture economics. My study highlights the importance of elucidating species-specific requirements through larval ontogeny and the need to adapt larval protocols to meet the demands of the rapidly developing larvae.

4.7 Acknowledgements

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Chapter 5. Ontogeny of the retina and visual pigments in larval and juvenile southern bluefin tuna, *Thunnus maccoyii*, and yellowtail kingfish, *Seriola lalandi*: Implications for feeding

5.1 Abstract

The visual development of southern bluefin tuna, *Thunnus maccoyii,* and yellowtail kingfish, *Seriola lalandi*, larvae was investigated to identify developmental sequences and any species-specific retinal adaptations that may have implications for understanding both the requirements for culture and larval ecology in the wild. The study included a histological examination of retinal anatomy, microspectrophotometry of retinal photoreceptors and behavioural analysis of feeding performance. First-feeding *T. maccoyii* and *S. lalandi* (3.4 ± 0.2 mm and 4.5 ± 0.1 mm standard length, respectively) possessed only single cone photoreceptors and, based on the spacing of adjacent photoreceptors and focal length, had similar maximum theoretical visual acuities (1.2 ± 0.1^0 and 1.1 ± 0.1^0, respectively). Rods and twin cones were present in the retina of *T. maccoyii* and *S. lalandi* by 21 days post-hatching (dph) (8.3 ± 0.2 mm and 10.0 ± 0.1 mm larval fork length, respectively) with cone mosaic patterns observed in *S. lalandi* at 21 dph but not until 30 dph in *T. maccoyii* (21.0 ± 4.2 mm larval fork length). Intra-retinal variations in the density of photoreceptors and ganglion cells were inferred from transverse sections of the retina. *Thunnus maccoyii* displayed high cell density in the ventral retinal region (cones, bipolar and horizontal cells), had half the convergence of cone cells on to ganglion cells compared to *S. lalandi*, double the available photon capture area (at first-feeding), and earlier development of retinal pigment epithelium (RPE) migration, which is associated with the retinomotor response. Microspectrophotometry showed that *T. maccoyii* had twin cone visual pigments maximally sensitive to light in the blue-green part of the spectrum (λ_{max} 494 nm, 507 nm and 524 nm), and behavioural experiments showed they fed preferentially at these wavelengths. In contrast, *S. lalandi*, displayed high cell density in the dorsal retinal region (rod and photoreceptor nuclei) and spectral sensitivity of the twin cones in the green spectral regions (λ_{max} 504 nm and 519 nm) with single cones also displaying violet sensitivity (λ_{max} 415 nm). Behavioural feeding experiments showed that *S. lalandi* had improved feeding under red light. High photopic acuity and photopic

sensitivity observed in *T. maccoyii* is hypothesised to be most likely associated with feeding in low light conditions, inferring possible adaptation to life in deeper oceanic waters and the ability to forage in low light intensities during dawn and dusk. In contrast, the *S. lalandi* visual apparatus provided high acuity for feeding under high light conditions in surface waters.

5.2 Introduction

Vision is considered the primary sense required for feeding in many marine finfish larvae, with the ability to see being ultimately dependent upon the capture of photons by the photoreceptors (cones and rods) via visual pigments, irrespective of the visual environment (Batty and Hoyt, 1995; Blaxter, 1986; Lythgoe and Partridge, 1991). Cones restrict feeding to conditions of high light intensity (photopic vision) and provide fish with visual acuity i.e., the detection of visual targets at distance with increasing perception of detail as the target moves closer to the eye (Fernald, 1989; Fritsches et al., 2003a). In contrast, rods increase the sensitivity of vision in low light enabling the detection of visual targets in lower light conditions (scotopic vision), and increase movement detection (Fernald, 1989). The full retinal complement, consisting of both cones and rods, provides fish with concurrent resolving power, acuity and sensitivity (Kotrschal et al., 1990).

In general, the type of photoreceptor present in first-feeding larval fish is restricted to a pure cone retina due to the physical constraints of a small eye (Kotrschal et al., 1990). Consequently, acuity is utilised at the expense of sensitivity. It is likely that visual acuity is of far greater importance to small pelagic larvae for the detection of prey than the ability to see in lower light levels. A small number of species, however, do show a duplex retina at the beginning of exogenous feeding, as seen in the spiny damsel fish, *Acanthochromis polyacanthus,* and the zebrafish, *Danio rerio* (Pankhurst et al., 2002; Raymond et al., 1995). However, the larvae of these species generally exhibit direct development, hatching as larger larvae in an advanced stage of morphological development, where the larval pelagic phase is reduced or absent (Balon, 1999). For the majority of fish, increased larval growth and larger eyes provide space for ongoing retinal development, although the visual capacity between species differs due to the retinal complement and the developmental rate (Blaxter, 1986; Johns, 1982; Kawamura et al., 2003; Kotrschal et al., 1990).

Previous behavioural studies investigating the effect of light intensity on the feeding response of larval southern bluefin tuna, *Thunnus maccoyii*, and yellowtail kingfish, *Seriola lalandi*, revealed distinct differences in the light intensities that promoted feeding between the two species (Chapters 3 and 4). Soon after first-feeding, high levels of feeding were observed in *T. maccoyii* at lower light intensities indicating either a preference for feeding in low light and/or a physiological sensitivity to low light conditions. This is unusual compared to *S. lalandi* larvae and many other marine finfish larvae, which display improved feeding response as light intensity increases (Blaxter, 1986; Carton, 2005; Cobcroft et al., 2001; Pankhurst and Hilder, 1998; Stuart and Drawbridge, 2011; Villamizar et al., 2011). This is likely an example of the evolution of the visual system of fishes to match their specific visual habitat among the diversity of aquatic habitats available (Guthrie and Muntz, 1993). As retinal morphology is restricted by size constraints, it would appear that larval fish possess the optimum retinal complement necessary for survival in their environment (Kotrschal et al., 1990). The preference for feeding in lower light conditions by *T. maccoyii* may reflect retinal adaptations that increase photon capture and neural processing necessary for target detection in conditions of low light. Conversely, the preference for feeding in high light environments by *S. lalandi* most likely reflects a visual adaptation for target detection in conditions where light intensity is not a limiting factor.

Previous histological studies have investigated the theoretical visual acuity in *S. lalandi*, however, photoreceptor development and the measurement of other retinal indices were not included (Carton and Vaughan, 2010; Miyagi et al., 2001). No histological study to my knowledge has examined the visual development in *T. maccoyii*, although the retinal development of the closely related Pacific bluefin tuna, *Thunnus orientalis*, has been investigated (Kawamura et al., 2003; Matsuura et al., 2010).

Bottlenecks in the commercial development and hatchery production of *T. maccoyii* and *S. lalandi* aquaculture industries include major mortality in the first two weeks of culture in *T. maccoyii* and high rates of malformation and lack of swimbladder inflation in *S. lalandi* (Battaglene and Cobcroft,

2007; Hutchinson, 2009). It is likely that the bottlenecks in the culture of both species are associated with early larval development, highlighting the need for a greater understanding of this stage (Cobcroft, 2013; Cobcroft et al., 2012; Hutchinson, 2009; Woolley et al., 2012; Woolley et al., 2013).

In order to identify mechanisms responsible for differences in photopic sensitivity between *T. maccoyii* and *S. lalandi* and to gain an understanding of species-specific visual ontogeny and rearing requirements, my study undertook a multidisciplinary approach to assessing visual ability. Investigation of visual capacity using several methods has the advantage of providing insights into retinal mechanisms underlying visual performance (Utne-Palm and Bowmaker, 2006). Histology of the retina reveals eye morphology, photoreceptor complement, photoreceptor diameter and arrangement, theoretical visual acuity and retinomotor response (Kotrschal et al., 1990; Pankhurst and Hilder, 1998). Microspectrophotometry allows measurement of the spectral absorbance of photoreceptor visual pigments (Shand et al., 2002; Ullmann et al., 2011), and behavioural experiments define larval functional visual ability (Carton, 2005; Cobcroft et al., 2001).

In the present study, I describe the development of the visual system in both *T. maccoyii* and *S. lalandi* larvae. Ontogeny of visual morphology is discussed in relation to the sequential development of photoreceptors, while also investigating cellular density, photoreceptor diameter, convergence of photoreceptors on to ganglion cells and the response of the retina to light and dark conditions (expressed as the retinomotor response) (Ali, 1959). Spectral sensitivity of larval visual pigments was measured directly by microspectrophotometry and indirectly through behavioural experiments which visually challenged early-feeding larvae to feed in different light spectra. The aim of the study was to compare the visual ontogeny of *T. maccoyii* and *S. lalandi* in order to identify developmental sequences and any species-specific retinal changes that may have implications for understanding larval ecology in the wild and the requirements for culture in the laboratory.

5.3 Materials and Methods

5.3.1 Embryo supply and incubation

Thunnus maccoyii and *S. lalandi* embryos were supplied by Clean Seas Tuna Ltd from their hatchery facility in Arno Bay, South Australia during January 2012. *Thunnus maccoyii* embryos were incubated in 450 L tanks at a stocking density of 200 eggs L^{-1} under ambient light conditions with water quality parameters maintained at 25.2 ± 0.5 ^{0}C (mean ± standard deviation here and throughout), dissolved oxygen 97.5 ± 2.3%, pH 7.8 ± 0.3, salinity 37.2 ± 0.1 ‰ and water exchange rate of 200% h^{-1}. *Seriola lalandi* embryos were incubated under similar conditions in 380 L tanks at a stocking density of 120 eggs L^{-1} under ambient light conditions with water quality parameters maintained at 22.3 ± 1.0 ^{0}C, dissolved oxygen 96.5 ± 2.0%, pH 8.1 ± 0.5, salinity 38.0 ± 0.1 ‰ with a water exchange rate of 20 % h^{-1}.

5.3.2 Larval rearing

5.3.2.1 Thunnus maccoyii larval rearing

Newly-hatched embryos were transferred into larviculture tanks immediately after hatching and reared in a green coloured, 13,500 L, cylindrical, flat-bottomed, fibreglass tank at a density of 3 ± 1 larvae L^{-1}. Light from a fluorescent light source, halogen source and ambient sunlight provided a photoperiod of 14: 10 (h L: D) with an average intensity of 60 µmol s^{-1} m^{-2} at the water surface. Light intensity was measured using a Li-Cor LI-250 light meter with an underwater flat quantum sensor LI-1925A (calibrated to air). Water quality parameters were maintained at 25.0 ± 0.1 ^{0}C, dissolved oxygen 108.0 ± 6.5%, salinity 37.0 ± 0.5 ‰ and pH of 8.0 ± 0.5. Water was exchanged at 22% h^{-1} and introduced at the base of the tank to provide an upwelling current. Larval standard length (SL) measured from the tip of upper jaw to the end of the notochord (n = 4) and eye diameter, measured on a dorso-ventral axis (n = 8), were recorded from hatch (0 days post-hatching (dph)) to 30 dph. Relative eye size ((eye diameter / standard length) x 100) was also recorded.

5.3.2.2. Seriola lalandi larval rearing

Newly-hatched embryos were transferred immediately after hatch and reared in a green coloured, 380 L, cylindro-conical fibreglass tank, at a density of 15 ± 1 larvae L^{-1}. A single fluorescent tube provided a photoperiod of 14: 10 (h L: D) with an intensity of 58.8 µmol s^{-1} m^{-2} at the water surface. Water quality parameters were maintained at 23.0 ± 2.0 ^{0}C, dissolved oxygen 95 ± 5%, salinity 38.0 ± 0.3 ‰ and pH of 8.1 ± 0.2. A single air-stone generated upwelling water current and water was exchanged at 20% h^{-1}. Larval standard length, eye diameter and relative eye size was recorded as for *T. maccoyii.*

5.3.2.3 Live feeds and turbidity

Large-strain rotifers, *Brachionus plicatilis*, enriched with Spirit® INVE, were added to the larviculture tanks from 3 dph and 2 dph for *T. maccoyii* and *S. lalandi*, respectively, at a density of 5 rotifers mL^{-1}. A turbidity of 2.5 ± 0.5 nephelometric turbidity units (NTU) from first-feeding was achieved through the addition of algal paste (Nanno 3600® Reed Mariculture, California). *Artemia* sp. (enriched with Ori-green® Skretting, a.m. feed and Spresso® INVE, p.m. feed) were added at a density of 0.1 metanauplii mL^{-1} from 8 dph onwards. From 15 dph *T. maccoyii* larvae were fed newly hatched *S. lalandi* larvae at a density of 0.5 larvae L^{-1}, increasing to 25 larvae L^{-1} by 25 dph.

5.3.3 Larval retinal histology

Ontogeny of visual morphology and the retinomotor response was determined through histology. Prior sampling of *T. maccoyii* larvae from commercial culture tanks (under normal light conditions) revealed unusually large quantities of retinal pigmentation when examined by histological analysis, preventing accurate quantification of individual photoreceptors. Consequently, retinal cell morphology for both species was determined from dark-adapted larvae, where larvae were exposed to two hours of total darkness prior to euthanasia by anaesthetic (0.06% 2-phenoxyethanol). Two larvae of each species from an age series (3, 9, 12,

15, 21 and 30 dph) were sampled for histological analysis. Larvae were fixed in 5% glutaraldehyde in phosphate buffer 0.1M, (pH 7.4, containing 20 gL^{-1} sucrose) at 4 ^{0}C for 24 h then washed three times for 10 minutes in 0.1M phosphate buffer solution (pH 7.4) prior to storage in 70% ethanol. Bones were decalcified in larger larvae (>15 dph) by soaking in 10% formic acid solution for 24 h, then washed three times for 10 minutes in a 0.1 M phosphate buffer solution (ph 7.4) prior to storage in 70% ethanol. Samples were then dehydrated through an ascending series of ethanol solutions (i.e., 90% and 100%) with 10 minute in each solution. Larvae were embedded in glycol methacrylate resin (JB4, Agar Scientific Ltd, UK) and serially sectioned at 2.0 µm in the transverse plane using a Microm (Heidelberg HM340) microtome fitted with a glass knife. Sections were air-dried and stained with Lee's Methylene Blue- Basic Fuchsin prior to mounting with a coverslip using TBS® Toluene-based liquid mounting medium. Sections were examined under light microscopy at a magnification of 400x and 1000x.

5.3.4 Retinal morphometric measurements

Retinal measurements were made on histological sections using image analysis software (LAS, EZ Leica applications). Cellular density measurements were taken from both eyes of each larva in the transverse section that had the largest eye diameter. One 50 µm transect was counted in the dorsal, medial and ventral retinal region of each eye. Cell layer thickness measurements were taken from three transects perpendicular to the retinal layers in the dorsal, medial and ventral region for each retina. The available light path was measured from the external limiting membrane to the base of the retinal pigment epithelium as defined by Pankhurst (1987). The inclusion of the retinal pigment epithelium in the calculation allows a truer measurement of the light path, than just the outer segments alone, as the tips of the outer segments can be covered by the retinal pigment epithelium and rod outer segments are known to extend almost to the base of the retinal pigment epithelium (N. Pankhurst pers. comm).

5.3.4.1 Linear cell density

Linear cone cell density (cones. 0.05 mm^{-1} retina) was determined by counting the prominent cone ellipsoids in the outer nuclear layer (ONL). Partial cells overlapping the transect on the left side were counted but not those overlapping on the right for consistency in counting. Linear rod cell density (rods. 0.05 mm^{-1} retina) was determined indirectly by counting the photoreceptor nuclei in the ONL and subtracting the number of cone ellipsoids present. Linear ganglion cell density (ganglion cells. 0.05 mm^{-1} retina) was determined by counting all cell nuclei present in a 0.05 mm transect within the ganglion cell layer.

5.3.4.2. Angular cell density

Angular cell density per ten minutes of visual arc on the retina (cones. 10' visual arc^{-1}) was calculated firstly using the formula from Neave (1984):

$$Ø = 2 \times \arctan \{h / (f - v)\}$$

where *Ø* is the angle an image subtends on the retinal transect, *h* is the transect length halved, *f* is the focal length calculated as *r* x 2.55 (lens radius multiplied by Matthiessen's ratio 2.55) and *v* is the distance from the external limiting membrane to the ganglion cell layer (*v* was only used for calculation of the angular density of ganglion cells as the ganglion cell layer is distorted in small larval eyes) (Poling and Fuiman, 1998; Vandermeer, 1994). Secondly, calculation of cells per 10' visual arc^{-1} was achieved by:

$$\text{Cells 10'visual arc} = ((\text{linear cell density} / (Ø \times 57.3)) /60) \times 10$$

Where *linear cell density* is cells per transect length (100 µm) *Ø* is angular cell density per ten minutes of visual arc on the retina, 57.3 is the conversion of radians to degrees, 60 converts to minutes and 10 provides the number of cells per 10 minutes of visual arc.

5.3.4.3 Theoretical visual acuity

Theoretical visual acuity (minimum separable angle) (MSA) was calculated as a function of cone density and focal length of the lens using the formula:

$$\alpha = \arcsin \{1.11 / (10d \times f)\}$$

where α is the MSA, f = focal length, d = cone cell density (cells. 0.1 mm^{-1} retina) 10 converts the linear density per 100 µm transect to cells/mm and 1.11 accommodates a 10% shrinkage factor during histological preparation (Neave, 1984).

5.3.4.4 Convergence of photoreceptors on to ganglion cells

The convergence of photoreceptors on to ganglion cells was determined by dividing the angular density of photoreceptors by the angular density of ganglion cells (due to the small size of the eyes) in the dorsal, medial and ventral retinal regions. A ratio higher than 1:1 indicated more than one photoreceptor converging on to each ganglion cell.

5.3.4.5 Cone cell diameter

Cone cell diameter was measured from transverse sections at the point where the largest, non-distorted circular ellipsoid section was encountered (n = 30 ellipsoids measured per species per age).

5.3.4.6 Available photon capture area

To determine the possible photon capture area in relation to ganglion cell convergence, the area of the cone (A) was calculated from mean ellipsoid radius (r), ($A = \pi r^2$) and divided by the convergence ratio of the cones on to ganglion cells.

5.3.5 Retinomotor response

To examine the development of the retinomotor response, larvae were randomly sampled from the culture tank and either dark-adapted for two hours in total darkness or light-adapted for two hours under a single broad spectrum fluorescent tube (NEC tri-phosphor 18 watt, FL20SSBR/ 18-HG,

T8) at a light intensity of 17 ± 2 µmol s^{-1} m^{-2}. After light or dark adaption, larvae were euthanised using 0.06% 2- phenoxyethanol prior to fixation, embedding and histological analysis using the methods previously described.

Retinal index (retinomotor response) was measured as a function of:

p/v and m/v

Where *p* = the thickness of the retinal pigment epithelium layer, *v* = the thickness from the outer edge of the retinal pigment epithelium to the external limiting membrane and *m* = the length of the cone myoid (Ali, 1959; Masuma et al., 2001; Torisawa et al., 2007). Three measurements were made for each retinal region (i.e., dorsal, medial and ventral region) from the left and right eye of each fish.

5.3.6 Microspectrophotometry

5.3.6.1 Retinal preservation for microspectrophotometry analysis

Larvae were collected from the rearing tank every third day from 3 dph to 30 dph and transferred into a 500 mL container before being placed into a 0.3 m^3 black box which eliminated all light. Larvae were left for two hours before being euthanised using 0.06% 2- phenoxyethanol and then dissected (in the dark) using an 860 nm infrared illuminator LED light source (All things Sales and Service, Western Australia) aided by a Sony HDR-CX500 handy cam operated in night mode with the screen external to the black box. Larvae were dissected in phosphate buffered saline (425 mosmol kg^{-1}, Oxoid BROO14G Dulbecco A). Retinal samples for small larvae (3 to 12 dph) were collected by severing the whole head for analysis. Samples for larger larvae (15 dph to 30 dph) were collected by individual eye dissection. The retinal samples were placed on a N^{o}.1 thickness 24 x 60 mm coverslip with the addition of one drop of freezing medium. The freezing medium was adapted from Cummings and Partridge (2001) and consisted of phosphate buffered saline (425 mosmol kg^{-1}, Oxoid BROO14G Dulbecco A), 10% Dextran 150,000 MW (SIGMA) and 15% Dextran 9,000 – 11,000 MW (SIGMA). The tissue was

macerated using a scalpel blade and/or teased apart with needles to dislodge individual photoreceptors from the surrounding tissue. The preparation was covered with a smaller coverslip and gentle pressure was applied for three seconds to remove surplus fluid prior to sealing with clear nail polish. Retinal preparations were placed in a light-proof container and then progressively cooled for two hours at 4 ^{0}C then two hours at -20 ^{0}C followed by storage at -80 ^{0}C. MSP samples were transported from Arno Bay, South Australia to the University of Western Australia on dry ice and then stored at -80 ^{0}C for later microspectrophotometry analysis.

5.3.6.2 Microspectrophotometry analysis

The spectral absorbance of photoreceptors was measured using a microspectrophotometer as previously described in Hart (2004) and Hart et al., (2011). In brief, spectral absorbance curves for the outer segments of rods, cones and twin cones were obtained using a single-beam wavelength-scanning microspectrophotometer. A pre-bleach sample scan of the outer segment recorded the amount of transmitted light across the spectrum from 330 nm to 800 nm. A baseline scan was then made in an area free of tissue close to the outer segment. The ratio between the transmission of light for the sample scan and baseline scan was converted to absorbance to create a pre-bleach spectrum. Afterwards, each outer segment was bleached with white light for two minutes and a sample scan and baseline scan were repeated to create a post-bleach spectrum. This provided confirmation that the visual pigments were photoliable. A bleaching difference spectrum was calculated by subtracting the post-bleach spectrum from the pre-bleach spectrum. The width and minimum length of each photoreceptor was measured. Definitive classification of photoreceptor type (i.e., single cone, twin cone and rod) was complicated as the freezing process and/or sourcing the photoreceptors from small larval fish meant the classical morphological appearance of rods and cones was not obvious. Twin cones were identified by the appearance of paired, morphologically similar photoreceptors; rods were identified as outer segments greater than 20 µm long and single cones were identified

in younger larval fish possessing a simplex retina. All remaining photoreceptors were categorised as unknown.

Only spectra that satisfied selection criteria as described in Hart et al., (1998) were retained for analysis. Absorbance spectra were analysed according to methods described in Hart (2004) and Hart, et al., (2011). *Thunnus maccoyii* data was fitted to an A1 template. A running average was fitted for *S. lalandi* as data appeared to be a mix of A1 and A2 pigments. In brief, data were smoothed by applying a variable-point unweighted running average and the wavelength of maximum absorbance (λ_{max}) estimated from a regression line fitted to the long wavelength limb of the data between 70% and 30% normalized maximum.

5.3.7 Short term feeding experiments

Short-term feeding experiments were conducted on the second day of feeding for both *T. maccoyii* (4 dph) and *S. lalandi* (3 dph), as previous *S. lalandi* experiments had revealed poor feeding responses in experimental aquaria on 2 dph. The experimental procedure for the short-term feeding experiments follows that reported in Chapters 2 and 3. In brief, the experimental design consisted of four replicates for each treatment, i.e., blue, white and red light, with 30 larvae placed in each replicate 3 L aquarium. A similar light intensity was provided across all spectral treatments (7.0 ± 1.0 µmol s^{-1} m^{-2}) with an Aquaillumination Sol Super blue module LED (C2 Development Inc., Iowa) providing blue light which emitted wavelengths between 450 nm and 480 nm, a single fluorescent tube (NEC tri-phosphor 18 watt, FL20SSBR/ 18-HG, T8) providing white light that emitted energy of wavelengths between 400 – 500 nm and 600 – 700 nm and a single fluorescent tube (Philips TL-D 18W/15 red) providing red light emitting wavelengths between 600 nm - 680 nm. Rotifers were added at a density of 2 rotifers mL^{-1}. Larvae were left undisturbed to feed for four hours then terminally sampled and microscopically examined for the presence of ingested rotifers. The proportion of feeding and intensity of feeding was recorded as mean ± sd (n = 4).

The proportion feeding was calculated as percentage of the population feeding:

Proportion feeding = feeding larvae / live larvae x 100

and feeding intensity, expressed as rotifers larva^{-1} min^{-1}, was calculated as:

Feeding intensity = rotifers ingested per feeding larva / 240 min

5.3.8 Adult retinae

A single adult retina was collected from a freshly dead *T. maccoyii* broodstock (127.1 kg, 179.7 cm fork length (FL), eye diameter of 50 mm) and a *S. lalandi* broodstock (10.1 kg, 98 cm FL, eye diameter of 28 mm). The fish were originally wild-caught and held in captivity at Clean Seas Tuna Ltd under artificial lighting regimes for at least 12 months. The retina was removed and fixed in 5% glutaraldehyde in phosphate buffer 0.1 M (pH 7.4, containing 20 g L^{-1} sucrose) for 24 h at 4 ^{0}C. After fixation the adult retinae were washed three times for 10 minutes in 0.1M phosphate buffer (pH 7.4) baths prior to storage in 70% ethanol. Histological processing followed the same procedure as outlined for larval retinal specimens, although sections were cut on the sagittal plane for the observation of cone mosaic patterns.

5.3.9 Statistical analysis

All statistics were analysed using SPSS statistics 19 (IBM). Statistical analysis on histological counts were analysed by two-way ANOVA (age and retinal region) and square root transformed where necessary to meet the assumptions of ANOVA. Data were evaluated for homogeneity of variance using Levene's test and a residual plot. Behavioural feeding experiments were analysed as either chi-square (proportion feeding) or one-way ANOVA (feeding intensity). Tukey's post hoc test was used to describe differences between means when the ANOVA was significant. Statistical significance accepted at $P \leq 0.05$ for all tests.

5.4 Results

5.4.1 Larval morphometrics

Thunnus maccoyii larvae were 3.1 ± 0.2 mm SL at hatch, 3.4 ± 0.2 mm SL at first-feeding and 21.0 ± 2.5 mm FL at 30 dph. Dorso-ventral eye and lens diameters (Fig. 1A) increased with age from 0.24 ± 0.1 mm and 0.070 ± 0.002 mm (respectively) at hatching, 0.26 ± 0.05 mm and 0.081 ± 0.018 mm at first-feeding and 2.81 ± 0.20 mm and 1.08 ± 0.06 mm at 30 dph (n = 4). Relative eye size increased from 7% at hatching to 12% at 30 dph.

Seriola lalandi larvae were 4.2 ± 0.1 mm SL at hatching, 4.5 ± 0.1 mm SL at first-feeding and 17.0 ± 0.1 mm FL at 30 dph (n = 30). Dorso-ventral eye and lens diameters (Fig. 1B) increased with age from 0.30 ± 0.01 mm and 0.090 ± 0.002 mm (respectively) at hatching to 0.31 ± 0.01 mm and 0.095 ± 0.002 mm at first-feeding and 2.00 ± 0.02 mm and 0.694 ± 0.050 mm at 30 dph (n = 4). Relative eye size increased from 7% at hatching to 11% at 30 dph.

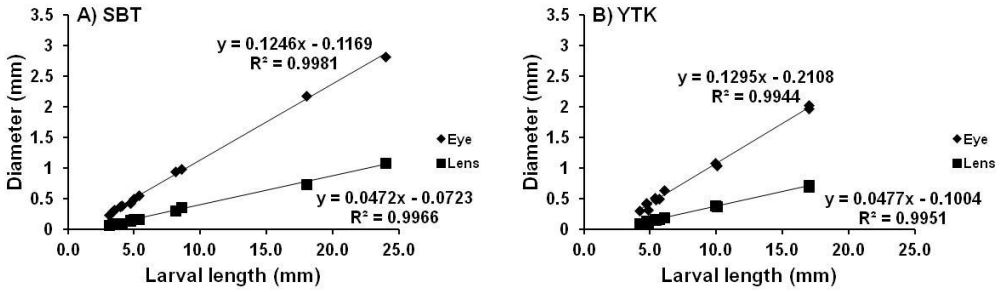

Figure 1. Change in eye and lens diameter of (A) *Thunnus maccoyii* (SBT) and (B) *Seriola lalandi* (YTK) with increasing body size. Mean ± sd, n = 8 (error bars are present but smaller than symbol size).

5.4.2 Retinal development

At hatch, the eyes of *T. maccoyii* and *S. lalandi* were simple hemispherical cups of undifferentiated, neuroepithelial tissue surrounding a central ball of undifferentiated tissue (the presumptive lens). By 1 dph, the retina had commenced differentiation into three distinct developing neural layers: the presumptive outer nuclear layer (ONL), inner nuclear layer (INL) and the ganglion cell layer (GCL). At first-feeding, *T. maccoyii* (Fig. 2A) and *S. lalandi* (Fig. 2B) had a crystalline lens with an outer cortical shell of fibre

cells. A fully differentiated single cone retina was present with pigmentation observed on the sclerad surface of the retina. At this stage the retina was presumed functional.

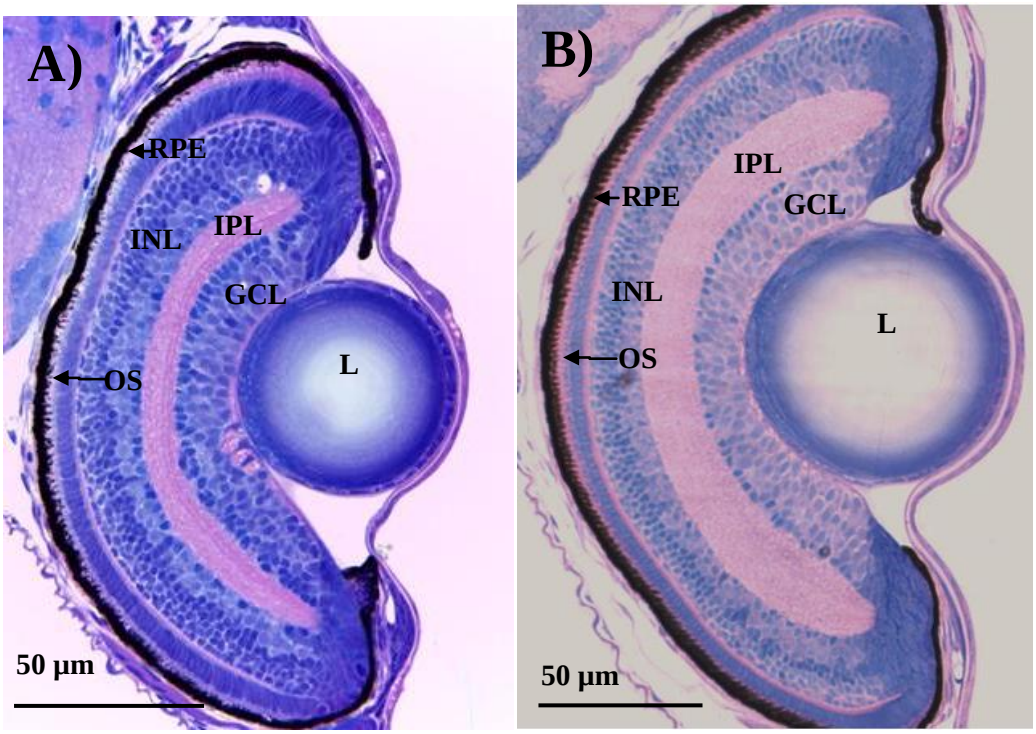

Figure 2. Photomicrographs of transverse sections through the eye of first-feeding (A) *Thunnus maccoyii* and (B) *Seriola lalandi*. Abbreviations: lens (L), ganglion cell layer (GCL), inner nuclear layer (INL), inner plexiform layer (IPL), outer segments (OS) and retinal pigment epithelium (RPE).

By 21 dph rod precursors were visible in *T. maccoyii* and *S. lalandi* as small, round, dark staining nuclei situated on the vitread border of the ONL. The development of twin cones was observed at 21 dph in both *T. maccoyii* (Fig. 3A) and *S. lalandi* (Fig. 3B) with a cone mosaic pattern obvious at 30 dph in *T. maccoyii* and 21 dph in *S. lalandi*.

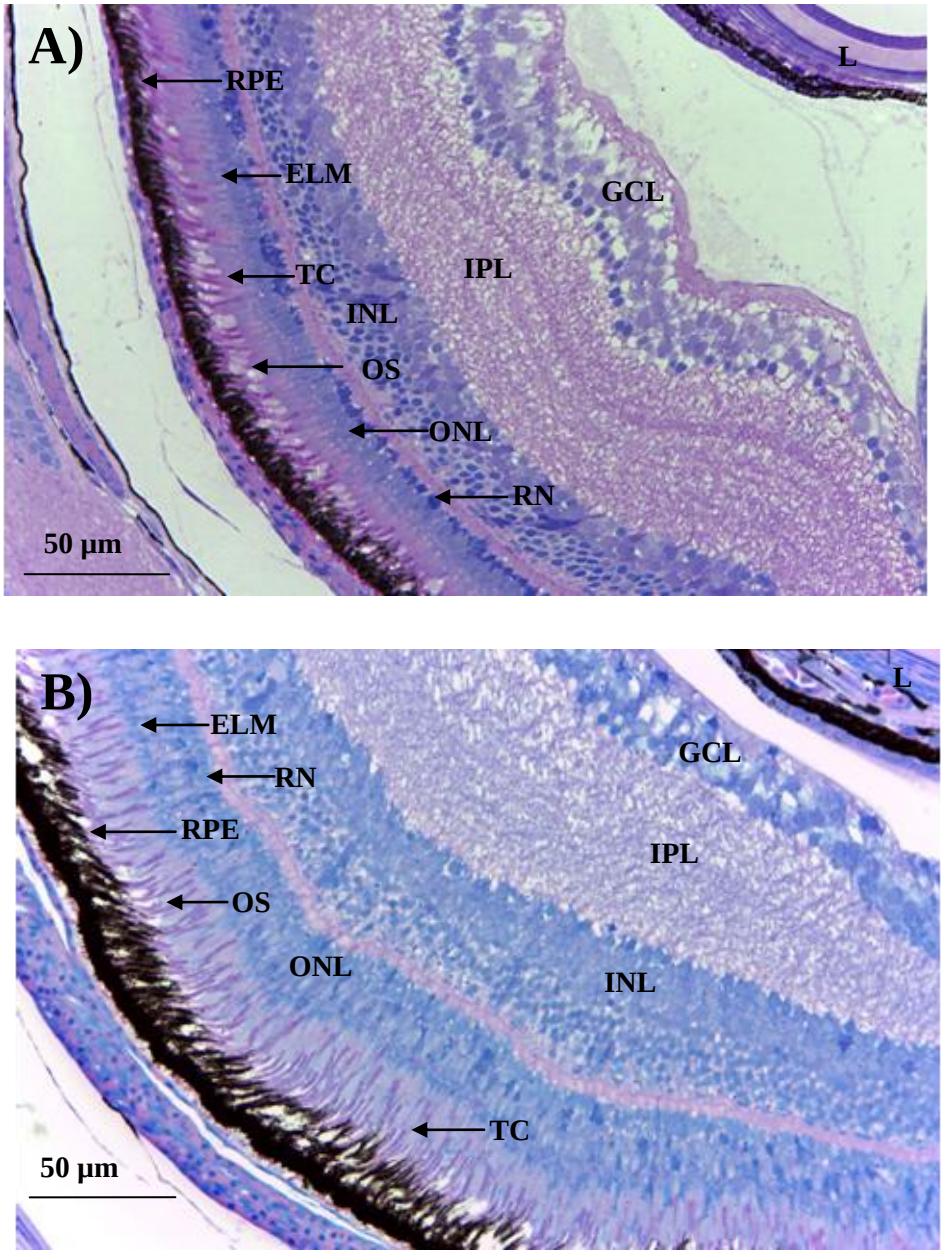

Figure 3. Photomicrographs of transverse sections through the eye of 21 dph (A) *Thunnus maccoyii* (B) *Seriola lalandi*. Abbreviations: lens (L), ganglion cell layer (GCL), inner nuclear layer (INL), inner plexiform layer (IPL), outer nuclear layer (ONL), rod nuclei (RN), twin cones (TC), outer segments (OS), external limiting membrane (ELM) and retinal pigment epithelium (RPE).

5.4.3 Retinal morphometrics

5.4.3.1 Linear cell density

Thunnus maccoyii and *S. lalandi* displayed a significant difference in the linear cell density, in response to retinal region, age and the interaction between retinal region and age (Appendix 1 and 2). Areas of high cell density were identified in the retinal regions of *T. maccoyii* and *S. lalandi*. Significantly, higher cone ($F_{2, 54} = 15.111$, $P < 0.001$) and horizontal cell density were observed in the ventral retinal region of *T. maccoyii* ($F_{2, 54} = 12.202$, $P < 0.001$). The remaining cells (bipolar, ganglion, photoreceptor nuclei and rods) all displayed significant interaction between retinal region and age (Appendix 2). With increasing age, there was a higher density of bipolar cells in the ventral region ($F_{10, 54} = 2.584$, $P = 0.012$), a higher density of ganglion ($F_{10, 54} = 6.376$, $P < 0.001$) and photoreceptor nuclei ($F_{10, 54} = 2.124$, $P = 0.038$) in the ventral and dorsal region compared to the medial region, and while rods also displayed a significant interaction ($F_{6, 54} = 8.064$, $P = 0.001$) a relationship between retinal area and cell density was not identified. In *S. lalandi*, cones were spread evenly through the retinal regions ($F_{2, 54} = 1.484$, $P = 0.236$), a high rod density was observed in the dorsal region ($F_{2, 54} = 10.470$, $P = 0.001$) and a significantly greater density of horizontal cells were present in the dorsal and medial region compared to the ventral region ($F_{2, 54} = 4.713$, $P = 0.013$). A significant effect of age was observed on photoreceptor nuclei, ganglion and bipolar cell densities. With increasing age a higher density of photoreceptor nuclei cells were observed in the dorsal region, with ganglion cells (at 15 dph, $F_{10, 54} = 2.511$, $P = 0.015$) and bipolar cells ($F_{10, 54} = 6.698$, $P < 0.001$) both displaying a significantly higher density of cells in the dorsal and ventral regions compared to the medial region.

Increasing larval age resulted in a decreased cell density as seen in *T. maccoyii* (3 to 30 dph), with a decrease in cones (45.1 ± 4.9 to 33.8 ± 5.4, cells 100 μm^{-1}, Fig. 4A), ganglion cells (93.1 ± 4.7 to 26.1 ± 6.1, cells 100 μm^{-1}), bipolar cells (145.8 ± 19.5 to 136.0 ± 51.5, cells 100 μm^{-1}) and horizontal cells (18.1 ± 4.6 to 9.5 ± 2.2, cells 100 μm^{-1}). This pattern also

occurred in *S. lalandi* (3 to 30 dph) with a decrease in cones (43.5 ± 5.9 to 37.2 ± 5.3, cells 100 μm^{-1}, Fig. 4C), ganglion cells (70.0 ± 11.7 to 14.5 ± 3.2, cells 100 μm^{-1}), bipolar cells (126.6 ± 5.9 to 124.8 ± 5.3, cells 100 μm^{-1}) and horizontal cells (13.5 ± 2.2 to 8.4 ± 2.2, cells 100 μm^{-1}).

In contrast, photoreceptor nuclei cell density increased in 21 to 30 dph *T. maccoyii* (46.1 ± 4.1 to 107.3 ± 21.9, cells 100 μm^{-1}) and *S. lalandi* (44.6 ± 4.2 to 92.0 ± 15.5, cells 100 μm^{-1}), which would identify the appearance of rods. Rod density increased from 22.9 ± 9.9 cells 100 μm^{-1} in 21 dph to 73.5 ± 19 cells 100 μm^{-1} 30 dph *T. maccoyii* larvae (Fig. 4B), and in *S. lalandi* from 26.9 ± 9.1 cells 100 μm^{-1} in 21 dph larvae to 54.1 ± 8.9 cells 100 μm^{-1} in 30 dph larvae (Fig. 4D).

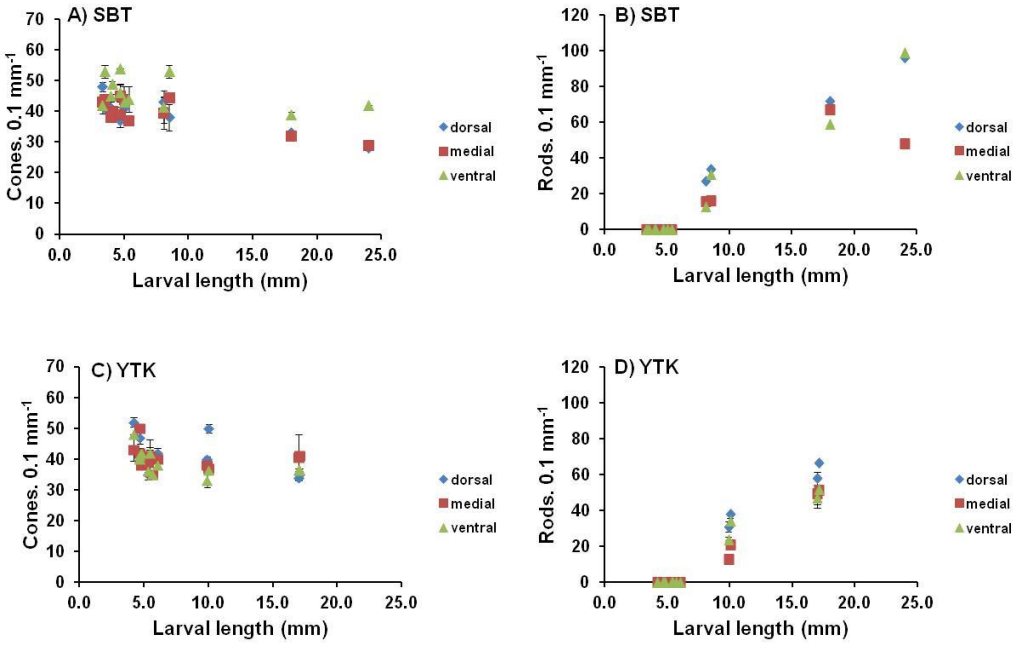

Figure 4. Change in the linear cell density of *Thunnus maccoyii* (SBT) (A) cones and (B) rods, and *Seriola lalandi* (YTK) (C) cones and (D) rods with increasing size. Values are mean ± sd, n = 2 larvae with one transect per eye for each retinal region.

5.4.3.2 Angular cell density

Higher angular cell density was observed in the retinal regions of both species that reflected results identified in the linear cell densities. The angular density of cones was significantly higher in the ventral region for *T. maccoyii* ($F_{2, 53} = 7.316$, $P = 0.002$), and evenly spread across the

retina for *S. lalandi* ($F_{2, 53} = 1.736$, $P = 0.186$). Rod angular cell density was even across the *T. maccoyii* retina ($F_{2, 53} = 2.119$, $P = 0.149$) and higher in the dorsal region for *S. lalandi* ($F_{2, 18} = 10.699$, $P < 0.001$). No high angular cell density area of photoreceptor nuclei was identified in *T. maccoyii* ($F_{2, 54} = 2.979$, $P = 0.059$), while *S. lalandi* displayed a significant interaction with higher photoreceptor nuclei angular cell density in the dorsal region with age ($F_{10, 54} = 4.510$, $P < 0.001$). *Thunnus maccoyii* ganglion angular cell density displayed a significant interaction between region and increasing age with two-way ANOVA although Tukey's post-hoc test did not distinguish significantly different groups ($F_{10, 54} = 2.036$, $P = 0.047$), whereas *S. lalandi* showed a greater angular cell density in the dorsal region compared to the ventral region ($F_{2, 54} = 14.809$, $P < 0.001$). The increase in eye size, with a corresponding greater focal length, observed with increasing age in both species, resulted in a greater number of photoreceptors per visual angle (Fig. 5).

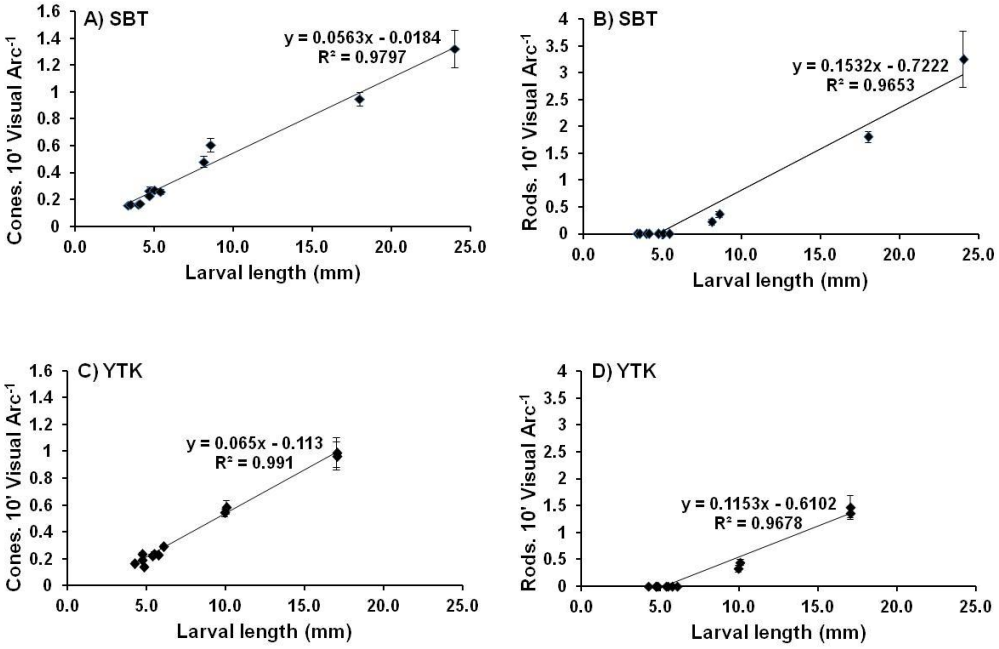

Figure 5. Change in the angular cell density of *Thunnus maccoyii* (SBT) (A) cones and (B) rods and *Seriola lalandi* (YTK) (C) cones and (D) rods with increasing age. Values are mean ± sd, n = 2 larvae with one transect per eye for each retinal region.

The angular cell density of *T. maccoyii* and *S. lalandi* significantly increased in all quantified retinal regions with greater age, as displayed in

Appendix 3 (angular cell density) and Appendix 4 (statistical summary of angular cell density).

5.4.3.3 Theoretical minimum separable angle (MSA)

Thunnus maccoyii exhibited significantly greater theoretical visual acuity in the ventral region ($F_{2, 54} = 9.178$, $P < 0.001$), whereas *S. lalandi* displayed no area of specialisation ($F_{2, 54} = 0.956$, $P = 0.391$). Greater acuity (i.e., smaller MSA) was observed with increasing larval age in both species ($F_{5, 54} = 236.962$, $P < 0.001$ for *T. maccoyii* and $F_{5, 54} = 216.180$, $P < 0.001$ for *S. lalandi*) (Figs 6A and 6B). No significant interaction between retinal region and age was observed.

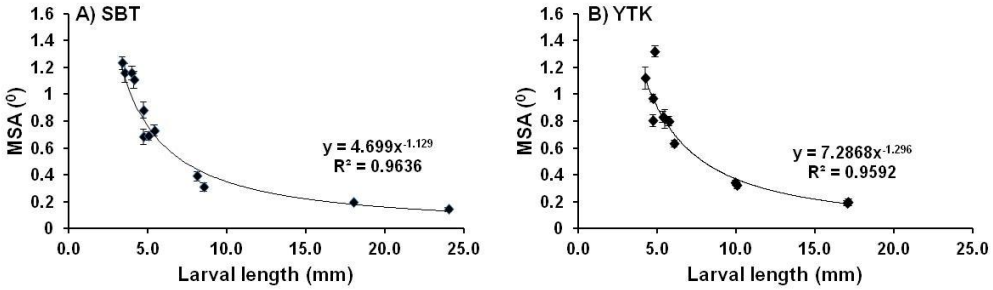

Figure 6. Change in MSA in (A) *Thunnus maccoyii* (SBT) and (B) *Seriola. lalandi* (YTK) (B) with increasing size. Values are mean ± sd, n = 2 larvae with one transect per eye for each retinal region.

5.4.3.4 Light path length and cell layer thickness

There was a significant interaction between retinal region and increasing age on the distance of the light path for both *T. maccoyii* and *S. lalandi* ($F_{10, 54} = 3.331$, $P = 0.002$ and $F_{10, 54} = 3.564$, $P = 0.001$, respectively). It was apparent that the distance of the light path was dependent on age. In *T. maccoyii* larvae younger than 15 dph the light path distance was similar, with a notable increase in distance observed between 15 dph and 21 dph larvae (more than double). The dorsal and ventral retinal regions had a significantly longer light path at 30 dph than equivalent regions at 21 dph (Figs 7A-7C). In contrast, *S. lalandi* displayed a longer light path in the dorsal and medial retinal regions at 15 dph, although as age increased (21 and 30 dph) only the dorsal region exhibited a significantly longer path. In

general, the overall cell layers were thicker in older larvae and the light path was longest in the dorsal region (Figs 7D-7F) (Appendix 5).

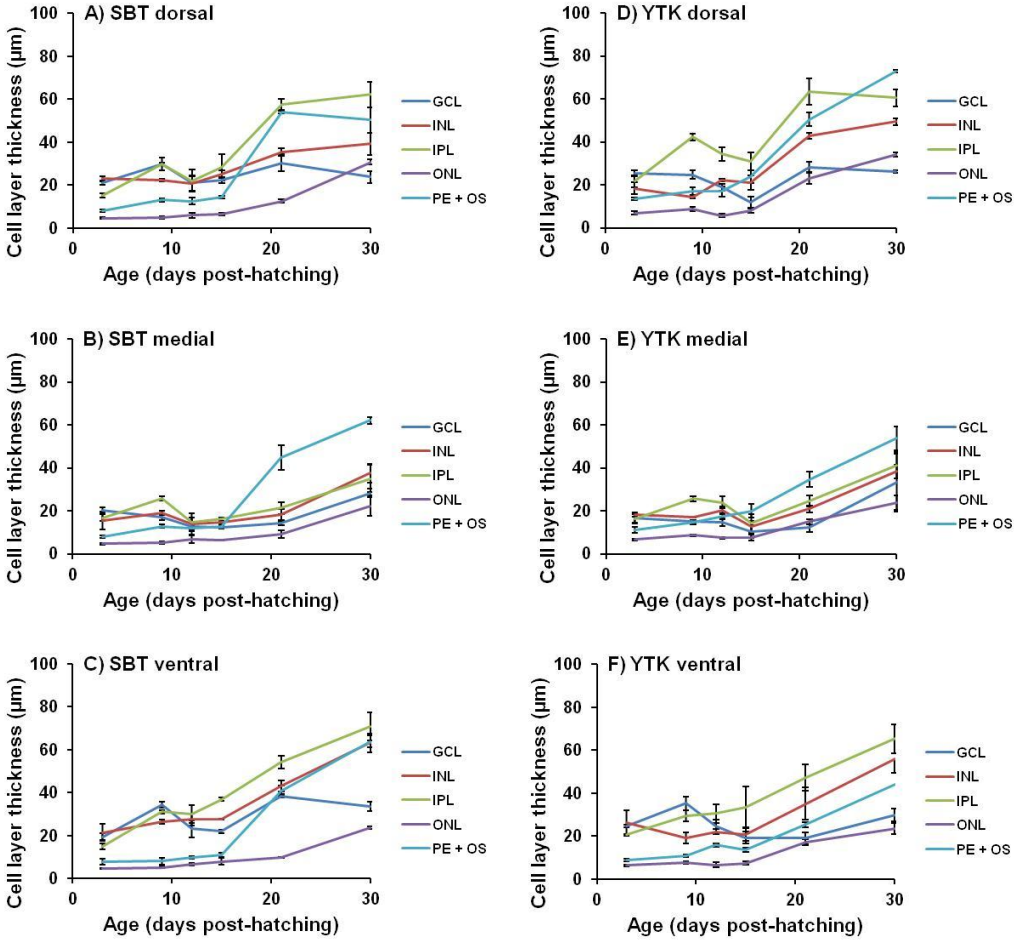

Figure 7. The change in cell layer thickness with increasing age in the retina of *Thunnus maccoyii* (SBT) in the (A) dorsal, (B) medial and (C) ventral region, and in *Seriola lalandi* (YTK) in the (D) dorsal, (E) medial and (F) ventral region. Abbreviations: ganglion cell layer (GCL), inner nuclear layer (INL), inner plexiform layer (IPL), outer nuclear layer (ONL) and light path (PE + OS). Values are mean ± sd, n = 2 larvae with three transects per eye for each retinal region.

5.4.3.5 Convergence of photoreceptors on to ganglion cells

The convergence of photoreceptors (P) on to ganglion cells (GC) was lower in *T. maccoyii* than *S. lalandi*. *Thunnus maccoyii* consistently displayed a low convergence of cones on to ganglion cells across all larval lengths (average 1.3: 1) with rods gradually increasing with larval size (Table 1). In contrast, the convergence of cones and rods in *S. lalandi* generally increased with greater larval size (Table 1), with a maximum

convergence ratio at 30 dph (P:GC) almost twice that of *T. maccoyii* (4.5 in *T. maccoyii* compared to 7.7 in *S. lalandi*).

Table 1. Convergence of *Thunnus maccoyii* and *Seriola lalandi* photoreceptors (ONL) (cones and presumptive rod nuclei) on to ganglion cells (GC), with increasing larval age. Values are mean ± sd.

Age dph	*Thunnus maccoyii*			*Seriola lalandi*		
	P:GC	Cones: GC	Rods: GC	P:GC	Cones: GC	Rods: GC
3	1.1 ± 0.1	1.1 ± 0.2	0	1.6 ± 0.3	1.6 ± 0.4	0
9	1.5 ± 0.3	1.2 ± 0.2	0	2.1 ± 1.0	2.1 ± 1.0	0
12	1.4 ± 0.8	1.3 ± 0.7	0	2.3 ± 0.4	2.3 ± 0.4	0
15	1.3 ± 0.3	1.3 ± 0.7	0	1.9 ± 0.8	1.9 ± 0.7	0
21	2.1 ± 0.6	1.3 ± 0.4	0.6 ± 0.3	4.0 ± 1.0	2.4 ± 0.6	1.6 ± 0.5
30	4.5 ± 1.1	1.4 ± 0.3	3.1 ± 0.9	7.7 ± 1.9	3.1 ± 1.0	4.5 ± 1.1

5.4.3.6 Cone cell diameter

There was a doubling in the enlargement of cone cell diameter with increasing larval length in *T. maccoyii* from 1.70 ± 0.12 µm at first-feeding to 3.53 ± 0.07 µm at 30 dph (Fig. 8A) and a similar trend in *S. lalandi* from 1.1 ± 0.1 µm at first-feeding to 2.8 ± 0.1 µm at 30 dph (Fig. 8B).

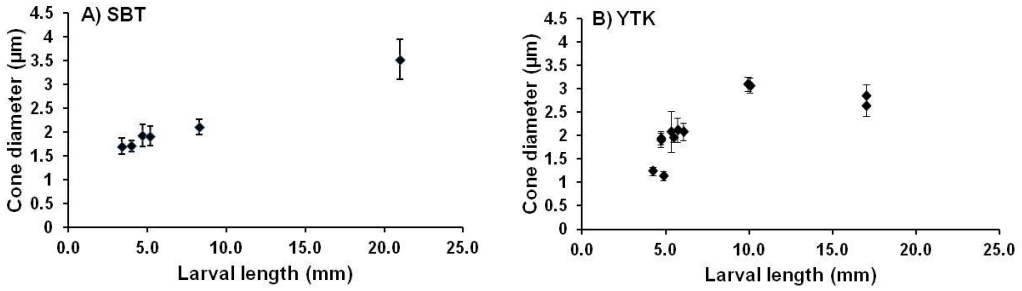

Figure 8. The change in cone cell diameter with increasing age of (A) *Thunnus maccoyii* (SBT), and (B) *Seriola lalandi* (YTK). Values are mean ± sd, n = 30.

5.4.3.7 Relative photon capture area per ganglion cell

The relative photon capture area was consistently higher in *T. maccoyii* compared to *S. lalandi*, most notably at first-feeding and 30 dph when relative photon capture area was three times greater (Fig. 9).

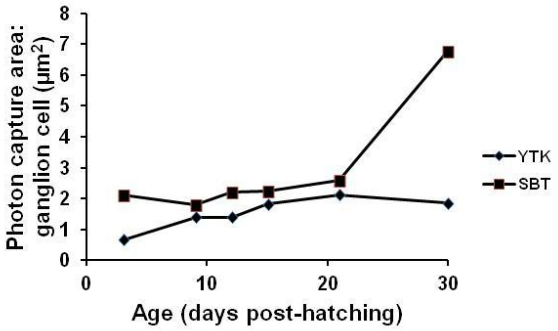

Figure 9. The relative photon capture area of *Thunnus maccoyii* (SBT) and *Seriola lalandi* (YTK) with increasing age. Values are mean ± sd, n = 2.

5.4.3.8 Cone mosaics

The photoreceptors of first-feeding *T. maccoyii* and *S. lalandi* were tightly packed single cones in a simple row arrangement (Figs 10A and 10B, respectively). The shift of cones to form a mosaic pattern was identified in 30 dph *T. maccoyii* and 21 dph *S. lalandi*. The temporal region of the retina of *T. maccoyii* displayed two mosaic patterns, a row mosaic and a regular square mosaic that were separated along the dorso-ventral axis. The square mosaic was primarily restricted to the retina closest to the midline of the body (internal), and the row mosaic was observed in the retina toward the external environment (away from the midline) (Fig. 11 A). In *S. lalandi* larvae, a single cone surrounded by four twin cones (a regular square mosaic), was observed distributed throughout the retina (Fig. 11B). Adult *T. maccoyii* did not display dual mosaic patterns, only row mosaics were evident (Fig. 12 A), whereas *S. lalandi* displayed the same regular, square, mosaic pattern as observed in juvenile fish.

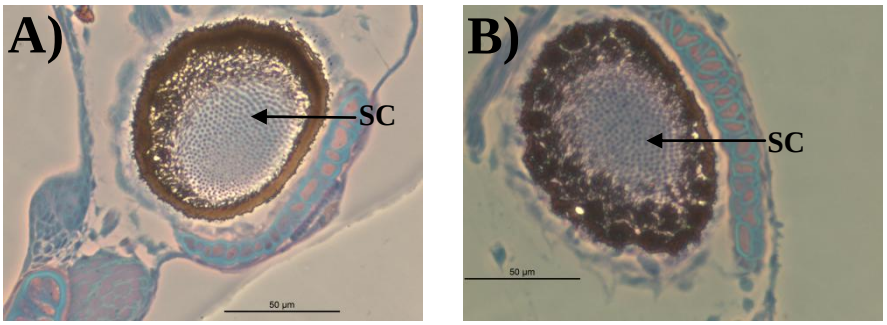

Figure 10. Photomicrograph of a transverse section through the eye with photoreceptor arrangement in (A) first-feeding *Thunnus maccoyii* and (B) first-feeding *Seriola lalandi*, both species showing tightly packed single cones in a simple row arrangement. Scale bars are 50 µm.

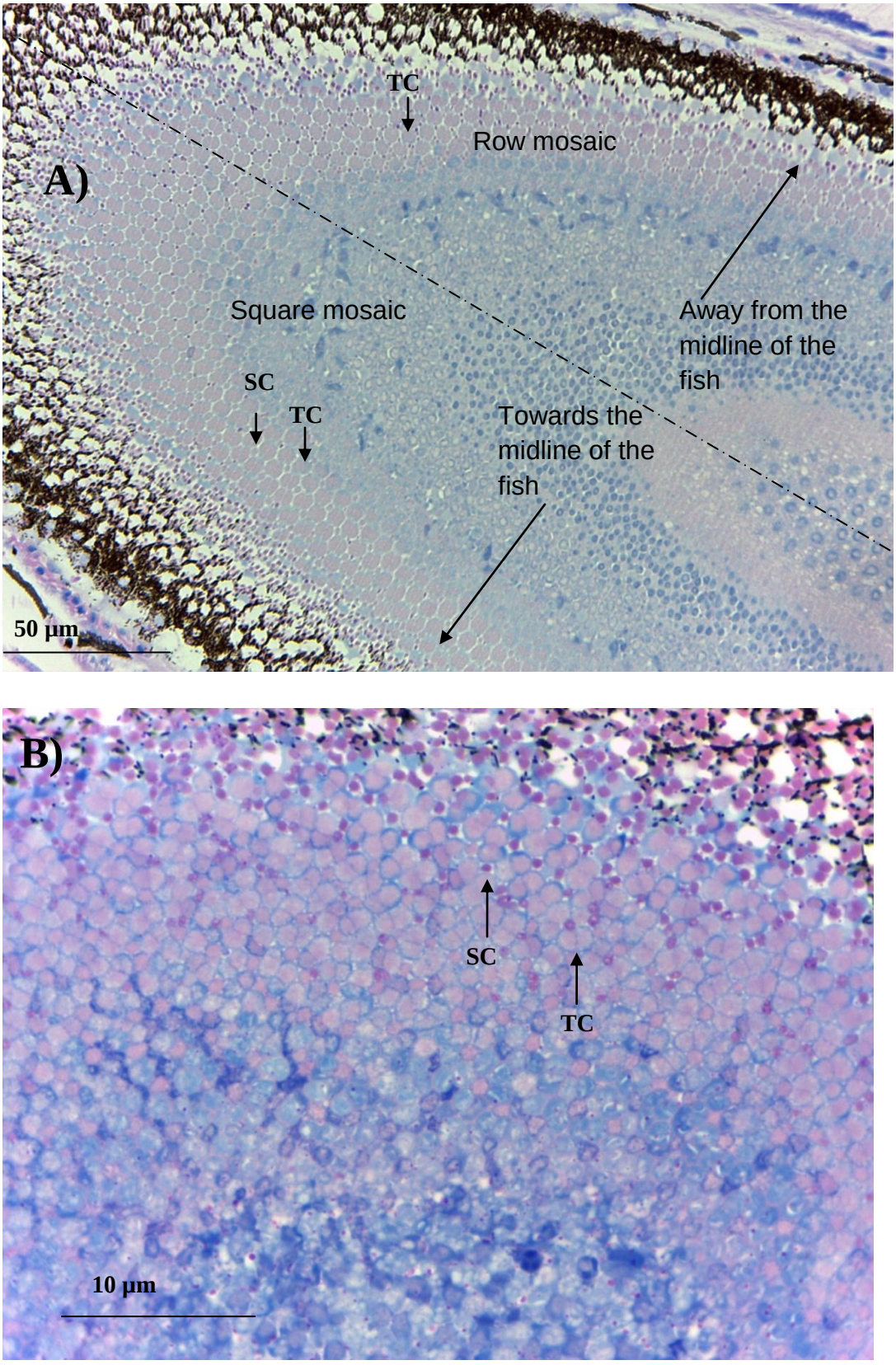

Figure 11. Mosaic pattern in (A) 30 dph *Thunnus maccoyii* where the retina had mainly a square mosaic on one half of the retina and mainly a row mosaic pattern on the other half of the retina (separated by the dashed line), and (B) the square mosaic pattern found in *Seriola lalandi.*

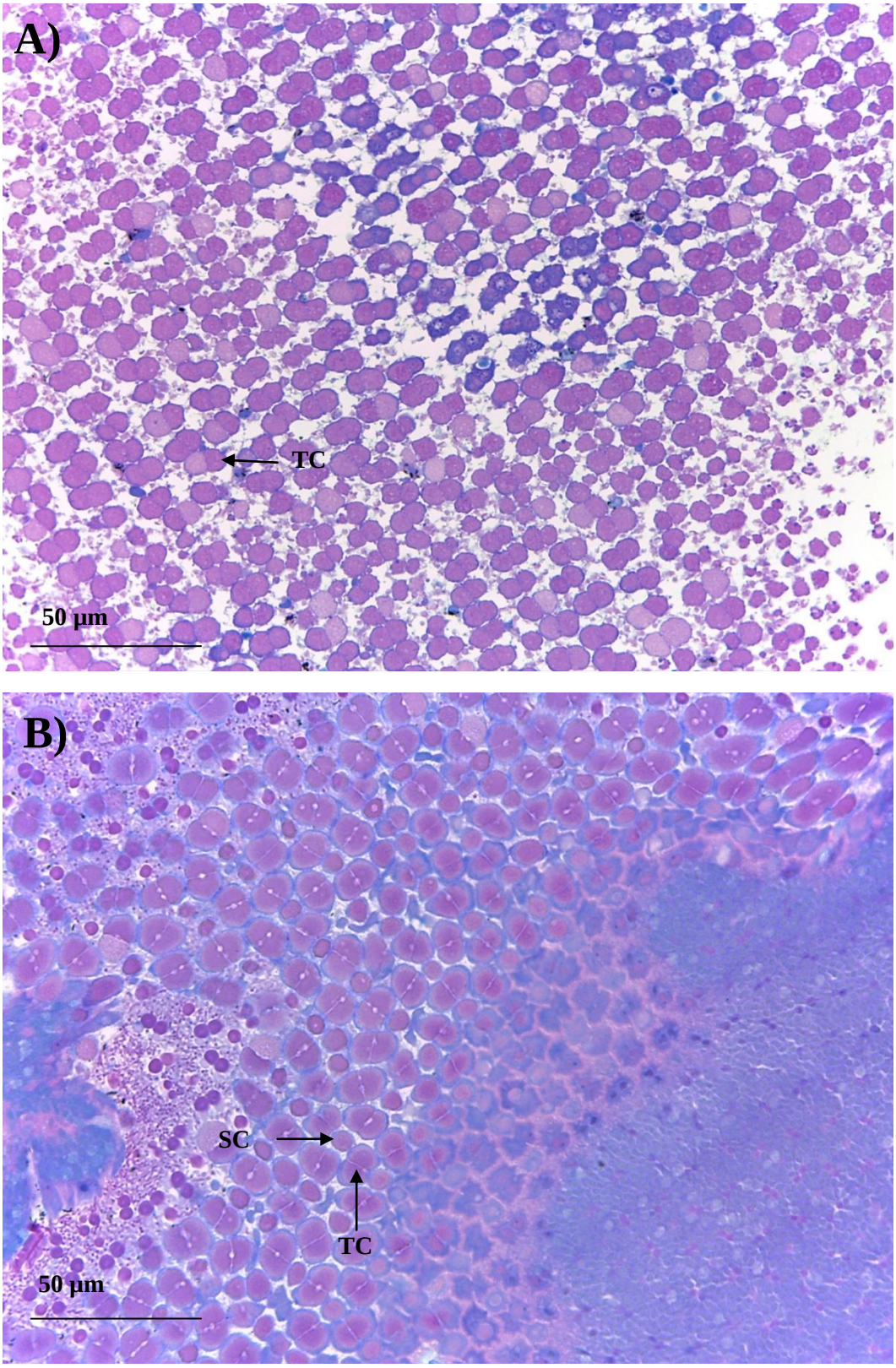

Figure 12. Photomicrograph of a sagittal section through the retina of (A) an adult *Thunnus maccoyii* showing twin cones present in parallel rows, and (B) adult *Seriola lalandi* with a regular square mosaic pattern. Abbreviations: Single cones (SC) and twin cones (TC).

5.4.3.9 Retinomotor response

Retinal pigment epithelium migration in response to light was initially observed in 4.5 mm SL *T. maccoyii* larvae (12 dph) and 10.0 mm SL *S. lalandi* larvae (21 dph) (Figs 13A and 13B, respectively) indicated by the higher p/v index found in light-adapted retinas compared to dark-adapted retinae.

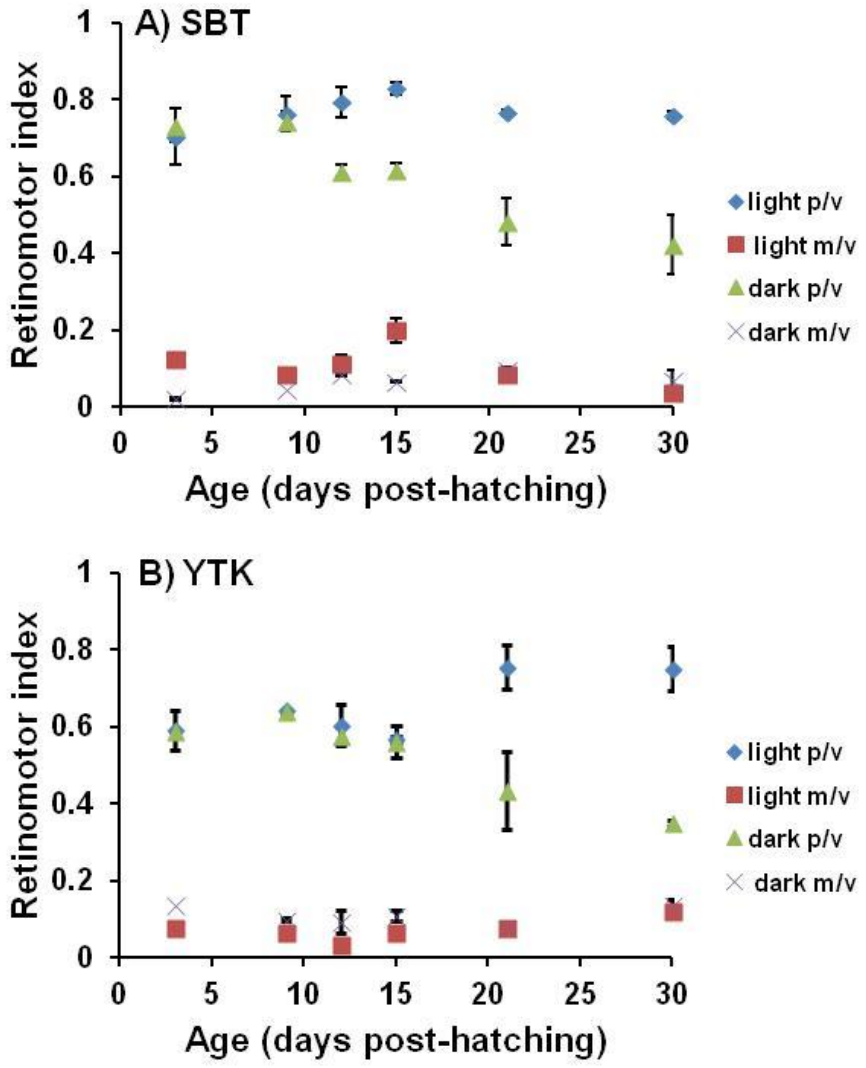

Figure 13. Retinomotor response in (A) *Thunnus maccoyii* (SBT) and (B) *Seriola lalandi* (YTK) where p/v and m/v indicate the migration of the retinal pigment epithelium and movement of the myoids (respectively), in light and dark conditions, with increasing larval size. Values are mean ± sd. Each measurement represents 9 transects from each eye of one fish.

Contraction and expansion of the retinal pigment epithelium was evident in photomicrographs of dark-adapted (Fig. 14 A) and light-adapted (Fig. 14

B) larvae. No contraction or expansion of cone myoids was observed in either *T. maccoyii* or *S. lalandi*.

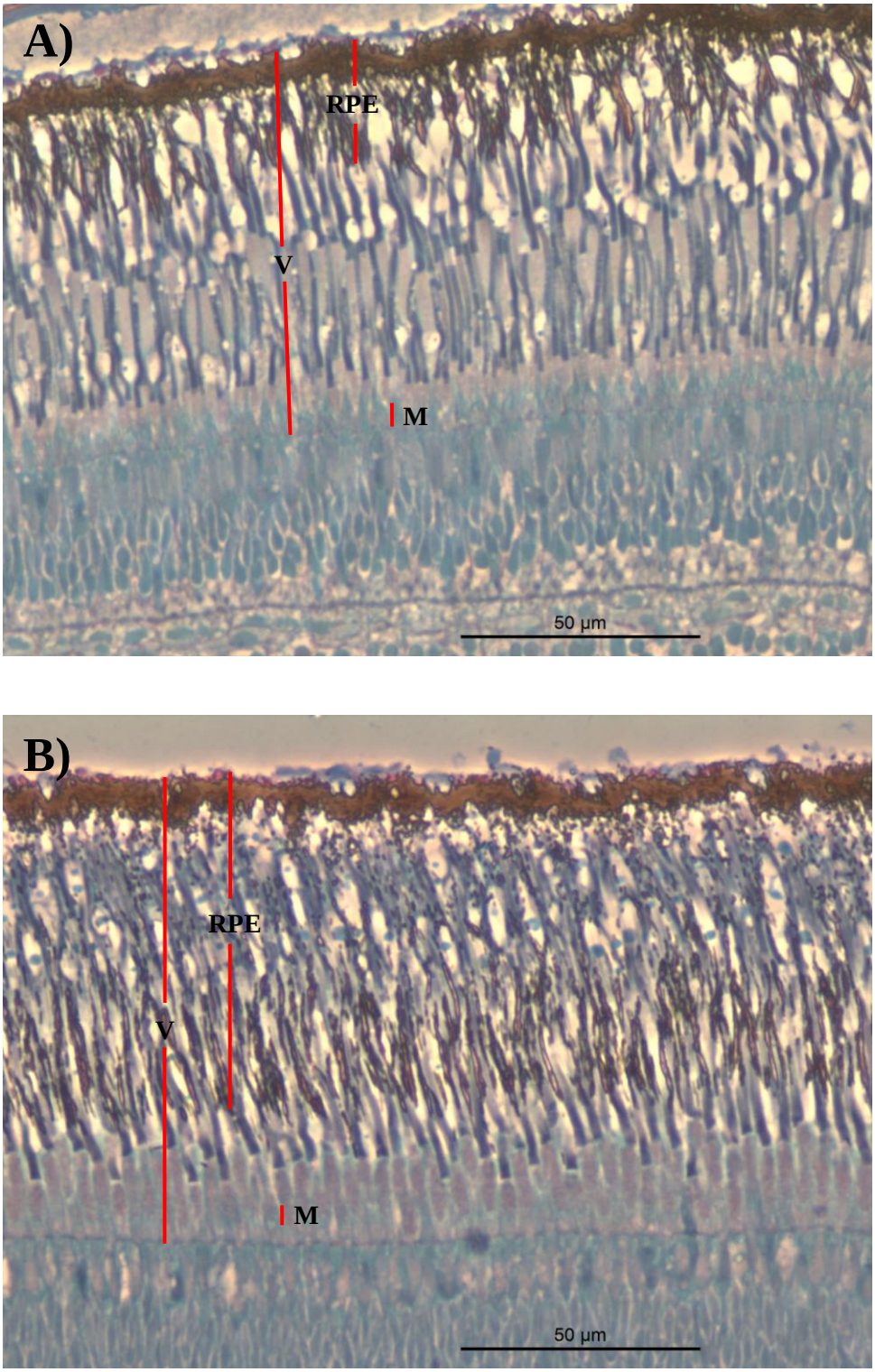

Figure 14. Photomicrograph of a transverse section through the retina of a (A) dark-adapted and (B) light-adapted 30 dph *Thunnus maccoyii*. Abbreviations: Retinal pigment epithelium (RPE), distance from the retinal pigment epithelium to the outer limiting membrane (V) and myoid length (M).

5.4.4 Behavioural feeding experiments

The proportion and intensity of feeding in *T. maccoyii* was significantly higher under blue light (Figs 15A and 15B) compared to red light (χ^2 = 24.748, df 2, P < 0.001 for proportion, and $F_{2, 9}$ = 6.062, P = 0.022, for intensity of feeding). In contrast, *S. lalandi* displayed the opposite result with a significant increase in the proportion and intensity of feeding under red light (Figs 15C and 15D) compared to white light, and no feeding observed under blue light (χ^2 = 31.135, df 2, P < 0.001 for proportion, and $F_{2, 9}$ = 12.739, P = 0.002 for intensity of feeding).

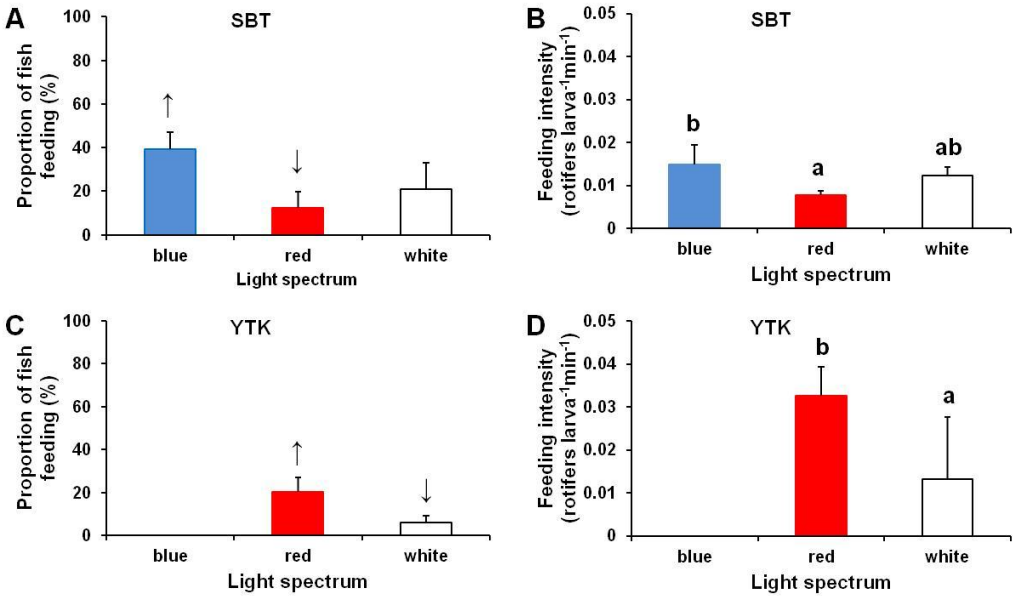

Figure 15. The feeding response of larval fish exposed to either blue, red or white light for *Thunnus maccoyii* (SBT) (A) proportion of larvae feeding, and (B) feeding intensity, and for *Seriola lalandi* (YTK) (C) the proportion of larvae feeding, and (D) feeding intensity. The arrows indicate treatments in which there were significantly more (↑) or less (↓) larvae feeding than expected (chi-square; P ≤ 0.05). Means sharing a common letter are not significantly different. Mean + sd, n = 4.

5.4.5 Microspectrophotometry

The range of λ_{max} values measured for visual pigments in *T. maccoyii* ranged from 478 nm to 546 nm (Fig. 16A, Table 2). Clustering of the spectral pigments was observed concentrated around 493 nm, 507 nm and 522 nm with over 52% of all values falling below λ_{max} 500 nm. In contrast, the range of λ_{max} in *S. lalandi* was 415 nm to 539 nm (Fig. 16B).

Clustering of the spectral pigments was observed around 415 nm, 497 nm, 513 nm, and 539 nm with 75% of all reading falling above λ_{max} 500 nm. The range of spectral sensitivity in *T. maccoyii* and *S. lalandi* remained constant over the investigated ages.

Table 2. Peak spectral absorbance sensitivity (λ_{max}) of *Thunnus maccoyii* and *Seriola lalandi* photoreceptor classes.

Wavelength (nm)	Rod	Cone	Twin cone	Unidentified
T. maccoyii	484 (n=3)	494 (n=4)	494 (n=5)	490 (n=8)
T. maccoyii		518 (n=3)	507 (n=4)	506 (n=3)
T. maccoyii		527 (n=4)	524 (n=3)	520 (n=3)
T. maccoyii				546 (n=1)
S. lalandi	502 (n=2)	415 (n=1)	504 (n=3)	496 (n=2)
S. lalandi		509 (n=2)	519 (n=3)	521 (n=3)
S. lalandi		533 (n=1)		

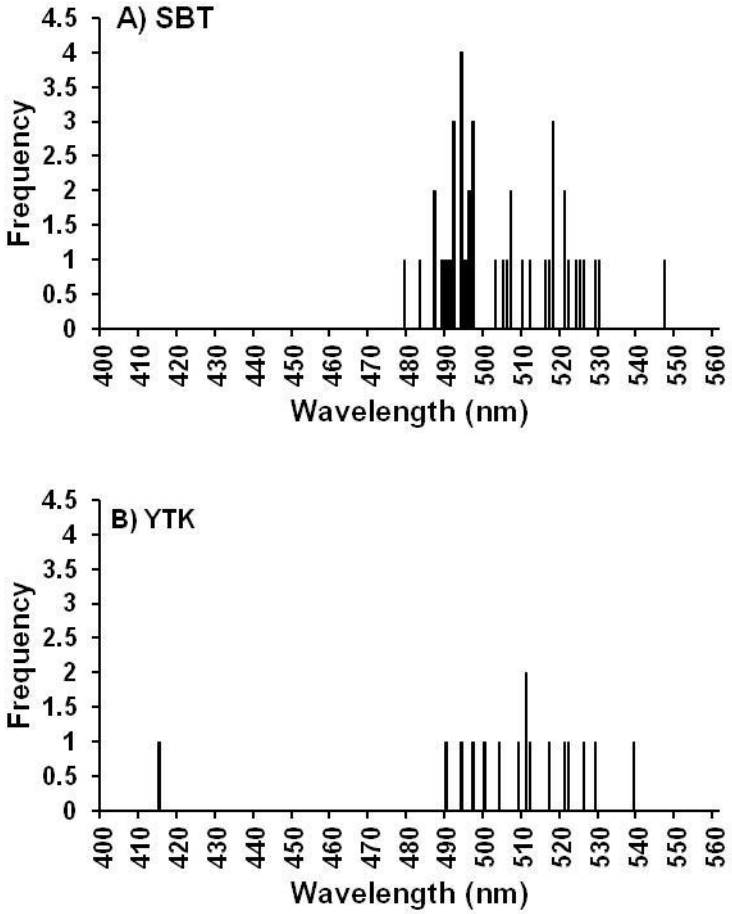

Figure 16. The λ_{max} spectral sensitivity of photoreceptors of (A) *Thunnus maccoyii* (SBT) and (B) *Seriola lalandi* (YTK).

5.5. Discussion

My study discovered fundamental differences between the retinal morphology and spectral sensitivity of *T. maccoyii* and *S. lalandi* larvae. Notably, the differences were displayed in the area of specialisation in the retina (area of high cone cell density), convergence of photoreceptors on to ganglion cells, available photon capture area, development of the retinomotor response and spectral sensitivity.

5.5.1 Morphology

Cones are associated with photopic acuity allowing the perception of detail, although this is at the expense of visual sensitivity in lower light conditions (scotopic vision) (Kotrschal et al., 1990). *Thunnus maccoyii* and *S. lalandi* follow the general template of visual development possessing a pure cone (simplex) retina at first-feeding and it is generally accepted that feeding success is dependent on relatively high light intensities. This is the case in first-feeding *S. lalandi*, but not in *T. maccoyii* where larvae fed equally well at lower light intensities and exhibited a decline in feeding performance at high light intensities with increasing age, indicating a basic difference in visual capacity between the species (Chapters 3 and 4). As *T. maccoyii* and *S. lalandi* display increased foraging ability, piscivory and the initiation of schooling behaviour at post-metamorphosis, an increase in visual capacity is required in order to complete these more visually complex tasks (Margulies, 1997; Torisawa et al., 2011). This was seen in 30 dph *T. maccoyii* and *S. lalandi* by the development of the full retinal complement (i.e., rods, twin cones and cone mosaics) and the rapid increase in visual acuity. Retinal development of the closely related *T. orientalis* shows a number of similarities with *T. maccoyii* (Kawamura et al., 2003; Matsuura et al., 2010). Cone development occurs in *T. orientalis* 25 to 60 h after hatching and rod development at 19 to 21 dph is associated with metamorphosis (Kawamura et al., 2003; Matsuura et al., 2010). Mosaics are first seen in 33 dph larvae (Kawamura et al., 2003). *Thunnus maccoyii* and *S. lalandi* display rapid visual development during

the first thirty days of life which highlights the importance of a highly developed visual system in post-metamorphic fish.

5.5.2 Linear cell density

Thunnus maccoyii displayed high cell density in the ventral retinal region with three of the six retinal cell types being present at a higher density. Vision is dependent on photon capture, so high cone density in the ventral region maximises the visual information available to the larvae coming from the dorsal visual axis (Naas et al., 1996). The higher horizontal cell density in the ventral region is associated with horizontal neural processing. This allows perception of movement (Kawamura and Tamura, 1973), which suggests that *T. maccoyii* larvae have increased detection of fast moving prey and predators in the dorsal visual plane. Consequently, it is not surprising that *T. maccoyii* have a high density of bipolar cells in the ventral region and a greater number of ganglion cells (compared to the medial region) as the amount of neural processing would be greatest in this area to maximise visual information to the brain. Acute visual function in the dorso-nasal plane has also been reported by Kawamura (2003) in *T. orientalis* and in three larval scombrid species by Margulies (1997). In comparison, high cell density in *S. lalandi* was located in the dorsal retinal region i.e., acute vision in the ventral visual field. Like *T. maccoyii*, the high density of horizontal cells would allow the improved perception of movement in the area of specialisation and the high rod density and subsequent nuclei density would promote enhanced scotopic sensitivity.

The high cell density recorded in the ventral retina of *T. maccoyii* and in the dorsal retina of *S. lalandi* throughout the 30 day developmental period, showed acute visual function in different and opposite visual fields. The upward facing visual field of *T. maccoyii* would most likely result in the detection of prey silhouetted against a brighter background and where the larvae are positioned in deeper waters. In contrast, *S. lalandi* with a downward facing visual field would detect prey, possibly highlighted by the scatter of light (halo effect), against a darker background with the larvae positioned in surface waters. In this case, high intensity, scattered light, as

seen in coastal surface waters, would provide the best illumination of prey for *S. lalandi.* In contrast, a sharp prey silhouette uninterrupted by light scatter, as seen in oligotrophic waters, would allow the greatest prey detection for *T. maccoyii.* If, as suspected, *T. maccoyii* are associated with low light conditions of deeper waters, the low convergence of photoreceptors onto ganglion cells consistently displayed throughout larval development would provide the larvae with greater visual acuity and sensitivity.

5.5.3 Visual acuity

The MSA reported in first-feeding *T. maccoyii* and *S. lalandi* (1.2 ± 0.1^0 and 1.1 ± 0.1^0, respectively) agree with other visual acuity studies of first-feeding tuna and yellowtail kingfish. Investigation into the acuity of three scombrid species revealed visual acuities ranging between 0.8^0 and 1.0^0 (Margulies, 1997) and a study investigating *S. lalandi* larvae recorded an acuity of 1.4^0 (Carton and Vaughan, 2010). Most larval marine fish generally display a MSA in the range between 1^0 and 2^0 (Blaxter and Jones, 1967; Carton and Vaughan, 2010; Margulies, 1997; Neave, 1984) as acuity is generally restricted by the size of the eye and the available focal length (Fernald, 1989; Margulies, 1997). The reduction in the MSA of *T. maccoyii* and *S. lalandi* coincided with increased eye growth and a slight reduction in cell density (due to retinal stretching) revealing that acuity is predominantly reliant on increasing focal length. This agrees with Margulies (1997) who suggests that focal length, not cone density, dominates the acuity relationship. As such, my study supports the principle that small fish will always have a higher MSA than larger fish of the same species as a function of the focal length of the lens. Consequently, smaller fish with a smaller visual field and limited detail perception need to be closer to the prey than larger fish to enable prey detection and subsequent capture. This is displayed behaviourally in marine fish larvae that commonly show a reactive distance of less than or equal to one body length (Blaxter, 1986). With growth the visual distance improves, as displayed in the spinycheek anemonefish, *Premnas*

biaculeatus, where larvae displayed a 63% increase in reactive distance between the ages of 3 dph and 10 dph (Job and Bellwood, 1996).

5.5.4 Retinomotor response

In the present study, the two species investigated exhibited significantly different ability to control the amount of light reaching the retina during the larval stage. The dual role of retinal pigment epithelium migration was evident in *T. maccoyii* larvae. Retinal pigment epithelium migration is generally associated with the development of rods, as these light sensitive photoreceptors require shading from high light conditions (Jobling, 1995; Neave, 1984). As retinal pigment epithelium migration was observed in *T. maccoyii* well before the development of rods this would suggest pigment migration plays an alternative role in the early life history of *T. maccoyii* larvae. Hodel et al. (2006) suggest that retinal pigment epithelium migration under photopic conditions may improve visual acuity, particularly during the early larval stages, as the cones are optically isolated by the interdigitating pigment, which prevents the scatter of light. As *T. maccoyii* have displayed preferential feeding at lower light intensities (Chapter 4) the early development of retinal pigment epithelium migration, may aid in increasing visual acuity in low light conditions. An alternative hypothesis may be that the retinal pigment epithelium migration is a visual adaptation to protect the larvae's light sensitive photoreceptors. The increase in *T. maccoyii* retinomotor index, associated with rod development, may aid in the protection and exposure of the light sensitive outer rod segments. The early pigment migration in *T. maccoyii* prior to the recruitment of rods differs to a number of tuna species where migration has only been observed with the presence of rods (Margulies, 1997). In *S. lalandi*, movement of retinal pigment epithelium was only observed in older larvae in response to the development of a duplex retina. Since *S. lalandi* larvae are associated with surface waters (Kolkovski and Sakakura, 2004; Smith, 1987), where relatively high light conditions prevail, the need for retinal pigment epithelium migration to increase photopic acuity is unlikely. Myoid movement was not observed in either species and photoreceptor

movement is reliant on the presence of a fully functional signal from mature rods perhaps not yet apparent in young larvae (Hodel et al., 2006).

My results indicate that *T. maccoyii* and *S. lalandi* have a well-adapted retinomotor response representative of their specific needs. The retinomotor response in *T. orientalis* and northern bluefin tuna, *Thunnus thynnus* has received intense investigation as it has been suggested that juveniles display visual disorientation during the transition from scotopic to photopic vision (Fukuda et al., 2010; Masuma et al., 2001; Torisawa et al., 2011; Torisawa et al., 2007). The consequence of this visual disorientation is mortality due to collision, as juvenile fish are unable to control their high power swimming ability in visually poor conditions. Collision mortality is also commonly experienced in juvenile *T. maccoyii* and is a major bottleneck in the production of hatchery fish entering the nursery. The explosive acceleration observed in tuna species generates sufficient power that impact against the tank wall often results in mortality (Ishibashi et al., 2013). My research indicates that *T. maccoyii* have well equipped visual apparatus for vision at lower light levels, at least until 30 dph, and tank collisions may be linked to their greater visual sensitivity particularly in a culture environment where visual cues such as torchlights can startle fish and initiate explosive swimming. However, the detection of low contrast solid objects, such as culture tank walls, may be more difficult than the detection of prey in low light, and contrasting grid patterns or lines may also aid in reducing juvenile *T. maccoyii* collision mortalities, as seen in *T. orientalis* culture (Ishibashi et al., 2013).

5.5.5 Cone mosaics

The development of cone mosaics in *T. maccoyii* and *S. lalandi* occurs at a time when both species develop piscivorous behaviour, which would require an increase in visual ability to detect faster moving prey. While both species exhibited a square mosaic pattern, which is associated with registering movement in all directions (Bathelt, 1970), the retina of *T. maccoyii* also possessed a row mosaic pattern. Row mosaics are characterised by the detection of movement in two directions and are

commonly associated with schooling fish which perceive a horizontal two-dimensional plane (Bathelt, 1970; Collin and Collin, 1999). Locket (1992) also suggests that a row mosaic may help in the capture of prey where a two-dimensional binocular axis would aid in the perception of depth. Retinal division characterised by two different mosaic patterns has been previously reported in adult striped marlin, *Tetrapturus audax*, (Fritsches et al., 2003b) and blue marlin, *Makaira nigricans*, (Fritsches et al., 2000) where delineation of the row and square mosaics was observed through the midline of the retina. These studies suggest this would allow the fish to use both mosaic pattern types. It is highly likely that *T. maccoyii* juveniles also utilise the two mosaic patterns to maximise visual information. While *S. lalandi* maintain the same mosaic pattern into adulthood, the shift to an all row mosaic pattern in *T. maccoyii* may imply increased importance in depth perception and peripheral motion detection associated with rapid swimming and spike dives (Willis et al., 2009).

5.5.6 Convergence

The convergence of photoreceptors on to ganglion cells defines the detail of the image that reaches the brain (Fritsches et al., 2003a; Pettigrew et al., 1988). The low convergence ratio observed in first-feeding *T. maccoyii* (1.1: 1.0), which continued throughout the larval life, would contribute to high visual acuity in *T. maccoyii* larvae. In comparison, *S. lalandi* with a convergence ratio of 1.6:1.0, would theoretically not experience the same degree of acuity as *T. maccoyii*. Both species at first-feeding displayed a relatively low convergence ratio and this has also been observed in first-feeding striped trumpeter, *Latris lineata* (Cobcroft, 2001). Kotrschal et al. (1990) explain that acuity in small larval eyes is reliant on a low cone to ganglion cell convergence. The large cone diameter of first-feeding *T. maccoyii* (twice that of *S. lalandi*), would also potentially double the photon capture area compared to *S. lalandi*, with a resultant increase in photopic sensitivity (Pankhurst and Butler, 1996; Vandermeer, 1994). The combination of low convergence ratio and high photon capture area increases the amount of visual information available to *T. maccoyii* compared to *S. lalandi*. However, this does not mean that *S. lalandi* are

visually inept. *Seriola lalandi* larvae and juveniles exhibit strong visual capacity, as displayed by the ability to feed over a broad range of abiotic and biotic conditions (Chapters 2, 3 and 4), and the development of piscivory and schooling behaviours, which both rely on strong visual capability (Margulies, 1997; Torisawa et al., 2011). What the combination of convergence ratios and photon capture area does show, is the high acuity (as a result of low convergence ratios) and increased sensitivity (due to available photon capture area), exhibited by *T. maccoyii*, particularly at first-feeding and after metamorphosis, highlighting the better visual capability of both *T. maccoyii* larvae and juveniles.

5.5.7 Spectral sensitivity

Thunnus maccoyii and *S. lalandi* larvae exhibit a broad spectral range (478 to 546 nm in *T. maccoyii* and 415 to 539 nm in *S. lalandi*) that is also seen in a number of other larval fish species (Britt et al., 2001; Loew et al., 2002). Studies by Loew et al. (2002), investigating the spectral changes in developing yellowfin tuna, *Thunnus albacares*, revealed a broad spectral range in larval fish that with increasing fish size (≥ 46 mm) condensed to reflect the narrow spectral range of adult fish. No restriction in spectral range was observed in larval *T. maccoyii* and *S. lalandi*, although fish were only investigated to 30 dph. It has been hypothesised that the broad spectral range of larval fish provides the opportunity for greater visual capacity over a broader light spectrum in the environment, with subsequent greater prey detection and increased predatory avoidance (Britt et al., 2001; Loew et al., 2002). Many studies have shown species-specific visual pigment complements (particularly twin cones) that reflect the ambient environment (Bowmaker, 1990; Loew and Lythgoe, 1978; Loew et al., 2002; Shand, 1993; Shand et al., 2002). The twin cones of *T. maccoyii* had spectral sensitivities at 494, 507 and 524 nm, with *S. lalandi* twin cone sensitivity observed at 504 and 519 nm. *Thunnus maccoyii* displayed spectral sensitivity encompassing the blue and green spectrum, whereas spectral sensitivity of *S. lalandi* was only observed in the green spectral region. Blue-green spectral sensitivity has also been observed in other juvenile and adult tuna species including *T. orientalis*, measured by

the expression of opsin genes in the retina, (Miyazaki et al., 2008), and by genome sequencing (Nakamura et al., 2013) and in *T. albacares*, measured by microspectrophotometry (Loew et al., 2002). In addition to the spectral sensitivity displayed in twin cones, the majority of *T. maccoyii* photoreceptors displayed spectral sensitivity below 500 nm in the blue spectrum (53%), whereas *S. lalandi* displayed the greatest spectral sensitivity above 500 nm in the green spectrum (82%). The presence of a single photoreceptor expressing pigment absorbing maximally in the violet range was also identified in *S. lalandi* larvae. The presence of ultra-violet and violet absorbing pigments has been identified in larvae of multiple species, with Britt et al. (2001) identifying 82% of the 22 species investigated possessing pigments absorbing in this range. Violet-sensitive pigments were not detected in *T. maccoyii*, as has been reported in *T. orientalis* (Miyazaki et al., 2008), although, as *T. albacares* are known to possess violet-sensitive pigments (Loew et al., 2002), it may be the pigments were not detected in my study, particularly if the prevalence of these cells was low. Ultra-violet and violet sensitive pigments are thought to increase the visibility of zooplankton, as violet light is reflected by the plankter increasing detection by larval fish (Lythgoe and Partridge, 1989).

The feeding performance of *T. maccoyii* and *S. lalandi* under coloured lights highlighted differences in the spectral sensitivities between the species. *Thunnus maccoyii* displayed significantly more feeding under light in the blue spectrum and *S. lalandi* displayed greater feeding in the red spectrum and no feeding under blue light. Consequently, *S. lalandi* larvae appear to not possess the visual apparatus or display a reduced willingness to feed under blue light. A number of species display greater feeding in red spectral environments including barramundi, *Lates calcarifer*, and *D. rerio* (Spence and Smith, 2008; Ullmann et al., 2011). Many shallow-dwelling species exhibit spectral sensitivity in the red spectrum and many larval fish exhibit an innate response to red coloured prey, as seen in carotenoid-rich zooplankton (Brown and Braithwaite, 2005; Smith et al., 2004; Spence and Smith, 2008). It has been suggested that the nutritional benefit from consuming carotenoid-rich prey may favour

the evolution of preference for red coloured prey (Olson and Owens, 1998). Investigation of visual pigment sensitivity by microspectrophotometry supported feeding preferences identified in the behavioural feeding experiments, although results should be interpreted cautiously due to the relatively small sample sizes.

It is well known that water acts like a chromatic filter as light passes through it, restricting the spectral bandwidth and the intensity with increasing depth (Jerlov, 1976). Light in the visible spectrum (380nm to 780 nm) is differentially absorbed as it enters the aquatic environment. Short wavelengths less than 390 nm (violet light) and wavelengths greater than 700 nm (red light) are rapidly absorbed compared to long wavelengths of 450 nm (blue light) (Villamizar et al., 2011). Coastal eutrophic waters with high particulate load exhibit rapid scatter and absorption of light and are characterised by maximum light transmission in the green-yellow spectrum. In contrast, oceanic oligotrophic waters with a low particulate load are typified by maximum light transmission in the blue spectrum. Spectral sensitivity observed in *T. maccoyii* and *S. lalandi* may be explained by larval adaptations to conditions encountered in the wild. As *T. maccoyii* larvae inhabit oligotrophic waters, where light is typified by maximum light transmission in the blue spectrum, the corresponding visual pigment complement with spectral sensitivity in the blue region would potentially increase the visual capabilities of the larvae (Davis et al., 1990; Davis et al., 1991; Jerlov, 1976; Young and Davis, 1990). Blue spectral light is also associated with light conditions at twilight and with increasing water depth (Munz and McFarlan, 1973). The broad spectral range displayed by *T. maccoyii* would potentially allow larvae, occurring in waters with uncertain prey availability, to forage over a greater range of depths and for longer periods of time (i.e., twilight and dusk) with the direct advantage of increased growth and survival. Increased growth reduces the time until juveniles become piscivorous, where a rapid acceleration in growth occurs, and the vulnerable larvae become top order predators. In contrast, *S. lalandi* larvae and juveniles take refuge in floating seaweed mats (Kolkovski and Sakakura, 2004; Smith, 1987) and may not require

the same adaption to such a wide range of environmental conditions for feeding and/or predator avoidance. Seriola *lalandi* larvae appear to inhabit upper surface layers occurring in coastal waters off New South Wales, Australia and in surface coastal waters ranging to 320 km offshore from Baja California, USA, where the larvae would most likely encounter light conditions consisting of high intensity, broad spectrum light (Jerlov, 1976; Smith, 1987; Sumida et al., 1985). This would help to explain the increased feeding observed in first-feeding *T. maccoyii* exposed to low light intensity and blue spectral light and the increased feeding observed in *S. lalandi* exposed to high light intensity and red spectral light.

5.6. Conclusion

The combination of behavioural feeding experiments and the identification of retinal ontogeny and spectral sensitivities provided a robust understanding of the visual ability of the larvae of *T. maccoyii* and *S. lalandi*. My study suggests *T. maccoyii*, particularly during their early development, and *S. lalandi* are adapted for growth in very different light environments. *Thunnus maccoyii* display retinal adaptations that are conducive to feeding in low light environments possibly associated with depth, whereas *S. lalandi* reveal adaptations to feeding in high light environments associated with surface, coastal waters. Consequently, light intensities utilised during culture should reflect the visual capacity of the larvae. My study has identified important factors in understanding the visual and subsequent culture requirements of the larvae, while also providing a possible insight into the ecology of the larvae in the wild. Suggested future research could identify the absolute sensitivity for *T. maccoyii* and *S. lalandi* by behavioural or electroretinograms to identify the light intensities where the rods saturate and the cones take over.

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Chapter 6. General discussion

6.1 Introductory comments

The overall aim of my thesis was to compare the visual development and visual capacity of southern bluefin tuna, *Thunnus maccoyii*, and yellowtail kingfish, *Seriola lalandi*, during larval ontogeny and to use this information to describe the best early larval rearing conditions to produce high quality seed stock. Functional visual ability, determined through behavioural experimentation, identified the effect of biotic and abiotic factors on first-feeding (Chapters 2 and 3). Important parameters identified in Chapters 2 and 3 were further investigated to determine the effect of larval development on feeding responses (Chapter 4). The key findings from the behavioural studies were that *T. maccoyii* are photopically more sensitive and have a greater overall feeding performance compared to *S. lalandi.* The importance of low light intensity during larval culture of *T. maccoyii* was highlighted and the necessity to optimise culture conditions during the early-life history of *S. lalandi* to maximise their feeding success. The basic difference in light intensity requirements between *T. maccoyii* and *S. lalandi*, identified in Chapter 4, drove the investigation into the spectral sensitivities of the species; that when combined with the examination of retinal ontogeny, identified fundamental visual differences between the species and helped to explain results of the behavioural feeding experiments (Chapter 5). My general discussion chapter explores the research findings from Chapter 2 through to Chapter 5, in the context of expanding the key findings and explaining the visual capacity of *T. maccoyii* and *S. lalandi* in relation to the requirements for culture. It also notes the potential relevance of the study to better understanding larval ecology in the wild.

6.2 Feeding experiments

6.2.1 First-feeding experiments

It is apparent that *T. maccoyii* easily made the transition from endogenous to exogenous feeding, in prey-rich waters, and appeared relatively unaffected by the surrounding visual environment. In general, *T. maccoyii*

displayed an increased ability to convert first-feeding opportunity into successful consumption displayed by the capacity to feed well over a wide range of environmental variables (five of the seven tested) (Fig. 1). Consequently, in a culture environment, successful feeding can be achieved over a broad range of conditions and mortality due to the failure to feed should be relatively low. Culture parameters that were identified to induce surface mortality included very bright light and air-generated turbulence and are important factors to be avoided in *T. maccoyii* culture. In contrast, the culture conditions must be more tightly controlled to initiate first-feeding in *S. lalandi*. While *S. lalandi* fed proficiently, they were restricted in feeding by a number of environmental variables (five of the seven tested) (Fig. 1).

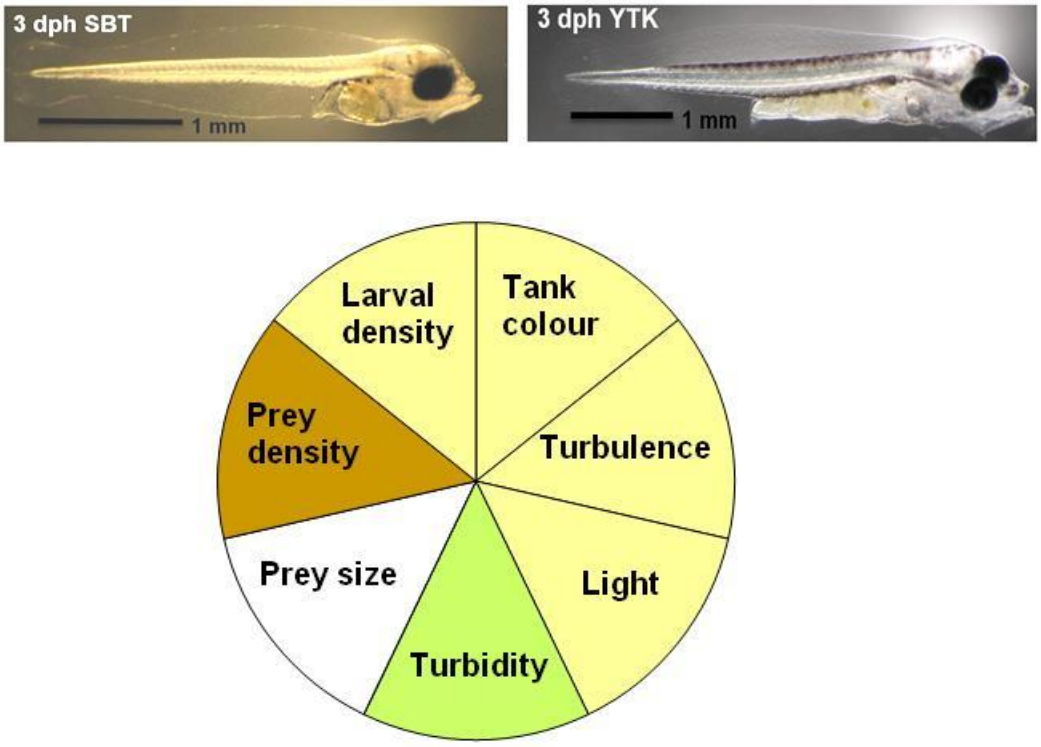

Figure 1. Photomicrographs of first feeding *Thunnus maccoyii* (SBT) and *Seriola lalandi* (YTK) and the abiotic and biotic factors that affect first-feeding. Significant factors affecting feeding are coloured green for *T. maccoyii*, yellow for *S. lalandi* and brown for both species. Unshaded factor signifies not significant. Photos by P. Hilder.

The knowledge that many variables affect the first-feeding success of *S. lalandi* larvae provides critical information for culture, highlighting the

importance of presenting larvae with optimum first-feeding conditions in order to promote feeding success.

Commercial larval rearing protocols at Clean Seas Tuna Ltd were based on empirical evidence where factors promoting successful larval culture were selectively employed in larviculture tanks. This trial and error approach required time and high investment in labour and capital resources, and limited the number of factors and their combinations that could be tested. The agreement in many factors promoting feeding, seen in both experimental and trial and error approaches highlights the usefulness of the short-term feeding experiments as an effective screening tool (Table 1). While many factors are in general agreement, my experimental approach indicated that low light intensity rearing may be an exciting breakthrough worthy of immediate testing in commercial tanks for *T. maccoyii*. For *S. lalandi,* the use of higher light intensities, as feeding response is improved, and it is likely to also support swimbladder inflation, and the use of tan or pink coloured tanks is also worthy of further consideration in long-term larviculture trials.

Table 1. Comparison of *Thunnus maccoyii* and *Seriola lalandi* first-feeding culture parameters used at Clean Seas Tuna Ltd (CST), Arno Bay, during 2010 and those recommended from my study 2012.

Culture parameter	*Thunnus maccoyii*		*Seriola lalandi*	
	CST	My study	CST	My study
Light µmol s^{-1} m^{-2} *	50*	0.4 to 9.9	126 to 203***	30 to 101 or higher
Turbidity NTU	2 to 3	0 to 2	2 to 3	3 to 24
Tank colour	Green	All tested	Green	Pink or tan
Turbulence	Medium	All tested	Low	Low
Prey density	10 mL^{-1}	25 mL^{-1}	2 to 5 mL^{-1}	25 mL^{-1}
Rotifer size	Large-strain	Large-strain	Large-strain	Large-strain
Larval density	20 L^{-1}	2 - 65 L^{-1}	80 - 100 L^{-1}	2 - 75 L^{-1}**

* Large variation in light levels due to natural sunlight with intensities up to 766 µmol s^{-1} m^{-2} recorded.** requires additional testing.***Converted from lx using Thimijan and Heins (1982). Data sourced from personal communication with CST staff and Cobcroft (2012; 2013).

6.2.2 Feeding experiments with 3, 6 and 9 dph larvae

The first-feeding experiments identified light intensity and prey density as factors warranting further investigation in longer-term experiments. While all the biotic and abiotic factors tested have important culture implications with on-going larval development, light intensity and prey density both offer potential long-term biological and economical efficiencies in culture. Comparison of the results from the initial first-feeding experiments of *T. maccoyii* and *S. lalandi* (Chapters 2 and 3) and the first-feeding results in the 3, 6 and 9 dph study (Chapter 4), demonstrated the same effects of light intensity and prey density, confirming the repeatability of the short-term feeding experiments.

The feeding performance of *T. maccoyii* and *S. lalandi* larvae highlights differences in their predatory ability and in the culture environment necessary to facilitate optimal feeding. While predatory ability in older *T. maccoyii* and *S. lalandi* larvae (i.e., 9 dph) is also a function of swimming ability, learned behaviours and ongoing sensory development (including mechanoreception, olfaction and gustation), the feeding proficiencies displayed are most likely are mainly a result of improved vision due to larval eye development (Chapter 5). The increasing photopic sensitivity, and prey consumption at low prey density, with increasing age in *T. maccoyii* indicates a developing visual system characterised by high sensitivity and acuity at a relatively early age. In comparison it appears that the visual system of *S. lalandi* larvae has evolved to function best in high photopic conditions providing acuity for prey detection but lacking high sensitivity.

6.3 Feeding experimental approach and standard parameters.

Short-term experiments provided valuable information into factors that affect feeding and mortality in *T. maccoyii* and *S. lalandi*. These experiments enabled the assessment of key variables over a relatively short time-frame, with good replication providing statistical power to detect significant differences. Importantly, the experiments allowed rapid identification of significant beneficial and detrimental parameters. This is

particularly important in the development of culture technology for new aquaculture species, such as *T. maccoyii*, where access to larvae for experiments is limited and especially if little information is available on larvae in culture or in the wild.

Experiments were designed to visually challenge the larvae and to "tease out" their visual and feeding capacity by testing a wide range of conditions for each experimental factor using a relatively low prey density (2 mL^{-1}). It was important to provide conditions that would allow significant feeding differences to be observed among the treatments, while at the same time neither allowing feeding to satiation nor totally inhibiting feeding. The conditions within each factor tested encompassed a range that extended well below, and well above current standard aquaculture practices for marine fish larvae. The experimental time-frame of four hours was determined from a preliminary experiment assessing the effect of feeding at two hours and four hours. A two-hour feeding period was determined to be too short a time frame to allow sufficient feeding, whereas the four-hour period delivered acceptable results. The remaining standard parameters; light intensity at 30 µmol s^{-1} m^{-1}, no turbulence, clear water and black aquaria were identified from the literature and commercial practice and chosen as variables that would be acceptable for both species. They were selected at the commencement of the study and were maintained throughout the experimental period for consistency and to avoid confounding the results. Experimental results indicated that some of the standard experimental variables chosen may not have been optimal for the feeding performance of the larvae e.g., high light intensity for *T. maccoyii* and black tanks for *S. lalandi*.

6.4 Spectral sensitivity

Thunnus maccoyii and *S. lalandi* are clearly affected by light conditions during larval rearing, both in terms of light intensity and light spectrum. The spectral λ_{max} exhibited in *T. maccoyii and S. lalandi* help explain the findings of the behavioural first-feeding experiments with coloured lights. This was apparent in the increased feeding response of first-feeding *T.*

maccoyii under blue light, while still possessing the ability to feed under broad spectrum white light and red light. A broad spectral sensitivity in *T. maccoyii* would increase available foraging grounds, improving the likelihood of prey detection in oligotrophic waters characterised by low or patchy distribution of prey (Chapter 5). In contrast, *S. lalandi* displayed increased feeding under red light, feeding in broad spectrum white light, and did not initiate feeding under blue light, which reflects the lack of blue-sensitive visual pigments. The complement of larval photopigment sensitivity and the spectral output from artificial light sources rapidly increases the likelihood of prey detection in culture. Villamizar et al. (2009) highlighted the importance of spectral quality, where the use of blue light in larval sea bass, *Dicentrarchus labrax*, rearing increased growth compared to white or red light and that spectral quality had a direct link to malformation incidence. Given the species-specific spectral sensitivities, the culture of larvae under artificial light sources that match the spectral range of the larvae has the potential to improve visual capacity and subsequent feeding opportunities. The use of blue light and red light, for *T. maccoyii* and *S. lalandi* respectively, is likely to improve visual performance and subsequent growth, particularly during their early life history, and is a non-expensive, easy factor to manipulate in culture and should be investigated in long-term culture trials.

Spectral sensitivity may also help to explain why first-feeding *T. maccoyii* had difficulty feeding in a high turbidity environment. Their visual pigment complement appears less sensitive to the dark-green spectrum. In contrast, the increased feeding ability of *S. lalandi* in highly turbid waters may indicate that first-feeding *S. lalandi* possess visually sensitive pigments in the green spectrum and/or *S. lalandi* benefit from improved prey detection against a contrasting background (Lythgoe and Partridge, 1989; Miner and Stein, 1993; Naas et al., 1992).

6.5 Retinal morphology

Examining the structure and ontogeny of eye development helped to explain some of the mechanisms behind the different feeding capacities and optimal larval rearing environment settings. While the timing in the sequential recruitment of photoreceptors was similar between *T. maccoyii* and *S. lalandi*, the species-specific retinal adaptations displayed by the larvae provided a possible explanation as to why *T. maccoyii* were capable of feeding in relatively low light intensity compared to the high light intensity required by *S. lalandi* for feeding. The observed photopic sensitivity of first-feeding *T. maccoyii* is likely due to the increased photon capture and visual information transfer as a result of large cones and low convergence of photoreceptors on to ganglion cells increasing the photopic sensitivity and photopic acuity (Lythgoe and Partridge 1991; Pankhurst and Butler 1996). Throughout the larval stage the low convergence ratio exhibited in *T. maccoyii* suggests a theoretically greater capacity to transfer visual information to the brain for image formation than *S. lalandi,* thereby providing greater visual acuity for the detection of prey as seen in the proficient feeding of *T. maccoyii* across the broad range of tested treatments.

The theoretical visual acuity reported in my study for first-feeding *T. maccoyii* and *S. lalandi* (1.2 ± 0.1^0 and 1.1 ± 0.1^0, respectively) agree with other visual acuity studies of first-feeding tuna and yellowtail kingfish (Table 2). In general marine fish larvae display a theoretical visual acuity in the range between 1^0 and 2^0 (Blaxter and Jones, 1967) as acuity is generally restricted by the size of the eye and the available focal length (Fernald, 1989; Margulies, 1997).

Table 2. Theoretical visual acuity in larval and juvenile fishes expressed as the minimum separable angle (sorted alphabetically by author)

Author	Species	Minimum separable angle
Carton and Vaughan, 2010	*Seriola lalandi* larvae	1.39^0 in 4 dph larvae and 0.97^0 in 7 dph larvae
Cobcroft, 2001	*Latris lineata* first-feeding onwards	No area of specialisation 1.1 to 1.6^0 at first feeding decreasing to 0.57 to 0.71^0 in 28 dph larvae
Haug et al., 2010	*Danio rerio* early larval period	3.1^0 in 5 dph larvae
Higgs and Fuiman, 1998	*Harengula jaguana* hatching	1.0^0*
Higgs and Fuiman, 1998	*Anchoa mitchilli* hatching	4.0^0*
Higgs and Fuiman, 1998	*Brevoortia tyrannus* hatching	2.1^0*
Margulies, 1997	*Euthynnus lineatus* first-feeding	Ventro-temporal specialisation 1^0
Margulies, 1997	*Auxix* spp. First-feeding	Ventro-temporal specialisation 1^0
Margulies, 1997	*Scomberomorus sierra* first-feeding	Ventro-temporal specialisation 0.8^0
Miyagi et al., 2001	*Seriola quinqeradiata* juveniles	0.41^0 in 15 mm larvae and 0.07^0 for 390 mm fish
My study	*Thunnus maccoyii* *3 to 30 dph*	1.2^0 in 3.4 mm larvae to 0.1^0 in 21.0 mm larvae
My study	*Seriola lalandi* *3 to 30 dph*	1.1^0 in 4.2 mm larvae to 0.2^0 in 17.0 mm larvae
Pankhurst et al., 1993	*Forsterygion varium*	1^0 8 min in 1 dph larvae and 54 min at 14 dph
Poling and Fuiman, 1997	*Micropogonias undulates* larvae	1.3^0 in 3.4 mm larvae and 0.4^0 in 10 mm larvae*
Poling and Fuiman, 1998	*Cynoscion nebulosus* early larval period	1.3^0*
Poling and Fuiman, 1998	*Micropogonias undulates* early larval period	1.3^0*
Poling and Fuiman, 1998	*Sciaenops ocellatus* early larval period	0.9^0*
Shand, 1997	*Apogon doederleine* post-settlement	0.8^0
Shand, 1997	*Pomacentrus moluccensis* pre-settlement	0.8^0
Shand, 1997	*Stethojulis strigiventer*	1.5^0

Shand, 1997	post-settlement *Upeneus tragula* pre-settlement	1^0
Torisawa et al., 2007a	*Thunnus orientalis*	0.3^0 at 30 dph to 0.04^0 at 1 year
Torisawa et al., 2011	*Thunnus orientalis* juveniles	0.4^0 at 25 dph to 0.09^0 at 55 dph

*estimate from graph

While the theoretical acuity has been documented for a number of species, few studies have experimentally tested the significance of retinal region and acuity in larvae as seen in Table 2. The retina in larval striped trumpeter, *Latris lineata*, displayed a constant acuity across all retinal regions (Cobcroft, 2001) which was similar to *S. lalandi* (Chapter 5). As larval fish are undeveloped and small, ontogeny of structural and physiological processes is restricted with development progressing first where the need is greatest (Kotrschal et al., 1990). This suggests that either *S. lalandi* larvae do not require an area of specialisation at this age, or more likely, the visual benefits associated with energy investment in eye growth (increasing eye size) outweigh the benefits of developing an area of high acuity. In contrast, high cone density in the ventro-temporal region was discovered in four scombrid species > 3.5 mm SL (Margulies, 1997; Torisawa et al., 2007). This agrees with the findings from my study for *T. maccoyii*. It appears that acute vision in the dorsal plane is important to *T. maccoyii* and other scombrids, indicating a need for increased prey and predator detection in an upward direction at an early age. Comparison of morphological and behavioural measures of visual acuity were attempted in this study, however, due to the small, transparent nature of the first-feeding larvae video vision could not provide sufficient detail to allow accurate measurement.

Behavioural experimentation revealed high light intensity induced significant mortality in *T. maccoyii* (Chapters 2 and 4)*;* and a notable difference in the retinal development was observed between the species (Chapter 5). *Thunnus maccoyii* displayed pigment migration at 12 dph, whereas migration was not observed in *S. lalandi* until 21 dph. As

significant mortality associated with high light intensity was no longer apparent in 12 dph *T. maccoyii*, it is highly likely that the development of retinal pigment epithelium migration is an important visual adaptation to provide shading and protection of the optically sensitive visual pigments from high light exposure during early larval life. Prior to the development of the retinomotor response, the only mechanism available to control light reaching the retina would be migration to deeper waters with lower light levels, which may possibly help to explain benthic migration observed in *T. maccoyii* (Cobcroft et al., 2012). The need to control the intensity of light reaching the retina in *T. maccoyii* during their early life history most likely indicates a physiological sensitivity to high light conditions from an early age.

6.6 Culture considerations

The development of a fully functional visual system in juvenile *T. maccoyii* and *S. lalandi* by 30 dph, not only highlights the importance that vision plays in the larval development of both species but also emphasises the need to match culture requirements of larval and juvenile fish exhibiting rapidly changing retinal morphology. *Thunnus maccoyii* and *S. lalandi* both display a number of culture bottlenecks where the light environment may contribute to aberrant behaviour or mortality (Table 3).

Table 3. Culture bottlenecks in the early life history of *Thunnus maccoyii* and *Seriola lalandi*.

Thunnus maccoyii	*Seriola lalandi*
Early surface mortality	First-feeding mortality
Lack of or malformation of the swimbladder	Lack of swim bladder inflation
Sinking mortality	Malformation (especially jaws)
Collision mortality	
Collision fractures and malformations	

Information obtained from Battaglene and Cobcroft, 2007; Cobcroft, 2013; Cobcroft et al., 2012 and Hutchinson, 2009.

My study shows that *T. maccoyii* and *S. lalandi* have different responses to light during culture and the elucidation of the correct light level and quality is critical in the production of quality seed fish. Evidence from my study provides potential explanations for the bottlenecks in *T. maccoyii* and *S. lalandi* production. I hypothesise that *T. maccoyii* larvae are actively avoiding high light conditions in culture because their visual apparatus is designed to function in low light conditions. High light conditions encountered by larvae are likely to result in decreased feeding and surface mortality during the early life history and promote benthic migration to the tank base in older larvae, with subsequent mortality. The sensitivity to light displayed by the larvae indicates the paramount importance of providing the correct visual environment for the culture of *T. maccoyii*, particularly during the early life history prior to the development of retinal pigment epithelium migration. In addition to the feeding experiments, behavioural observations of larvae within the long-term culture tanks used in my study supports the aversion to high light intensity of *T. maccoyii* larvae. Larvae were generally only observed near the tank surface in the top 10 cm of water at dawn and dusk and with increasing light intensity, larvae made a vertical migration towards the base of the culture tank. In early-feeding larvae this was particularly evident after the first addition of prey in the morning when larvae had fed and may have been related to increased body density, as seen in *S lalandi* where the ingestion of *Artemia* sp. significantly increases larval body density (Woolley et al., 2012). In older larvae, greater than 12 dph, larvae were observed less often, although, in cohorts exhibiting good larval behaviour and survival, larvae were seen in the late afternoon near the tank surface when there appeared to be a feeding peak.

Sinking mortality is a major problem in *T. maccoyii* and *T. orientalis* culture (Cobcroft et al., 2012; Masuma et al., 2011; Matsuura et al., 2010; Nakagawa et al., 2011; Sawada et al., 2005; Tanaka et al., 2009). Observation of *T. maccoyii* larvae in the culture tanks on a number of occasions revealed larvae situated just above the base of the tank with full guts actively maintaining position in an aggregation facing into the

oncoming water current. The larvae appeared to actively choose their position in the tank. Parameters which change with tank depth are light and pressure. Without discounting pressure, my study would indicate that larvae are making an active visual choice to reside in lower light conditions. The unfortunate side effect of this is that the tank base is associated with high detritus and bacterial loads that compromises larval health, resulting in mortality. In order to minimise larval migration to the tank base, relatively high turbulence was maintained in the culture tank, as is the practice in Japan for *T. orientalis* (Nakagawa et al., 2011; Tanaka et al., 2009). This potentially removes the control larvae have over their exposure to light, as the small larvae have difficulty in breaking free of the water current. It is highly likely that the continual exposure of the larvae to higher light intensities at the surface of the tank would contribute to larval stress, resulting in decreased feeding and/or surface mortality.

While collision mortality in *T. orientalis* is thought to occur due to poor scotopic vision (Ishibashi et al., 2009), the photopic sensitivity exhibited in larval *T. maccoyii* would indicate fish are not visually limited at low light intensity. While my study did not test the sensitivity of juvenile *T. maccoyii* in low light intensity conditions, histology results, namely the low convergence ratio of photoreceptors to ganglion cells and increasing cone cell diameter (in addition to the development of a duplex retina), would indicate juvenile *T. maccoyii* maintain increased sensitivity beyond the larval stage. It is more likely that juvenile fish are unable to control their newly acquired explosive swimming ability when “spooked” in a culture environment, although, the detection of a tank wall with minimal contrast in low light levels may be more challenging then detecting prey items in the water column. Collision mortality has been reduced in *T. orientalis* by modification of the visual environment, including the use of lights at night in sea cages and the addition of wall patterning or contrasting colours on the culture tank walls (Ishibashi et al., 2013; Ishibashi et al., 2009). Both methods may be helpful in minimising spooking and collision mortality in *T. maccoyii*, particularly during early juvenile development, as the young fish become accustomed to on-growing conditions in the culture environment.

In addition to the data collected in my study for *S. lalandi*, high light intensity has also been shown to significantly improve growth and swimbladder inflation (Carton, 2005; Stuart and Drawbridge, 2011; Woolley et al., 2012). The greatest growth and/or swimbladder inflation has been achieved at the highest tested light intensities indicating that the limit of light intensity may not have been achieved in previous studies (17 µmol s^{-1} m^{-2}, 590 µmol s^{-1} m^{-2}, and 275 µmol s^{-1} m^{-2}, respectively) and warrants further investigation. While high light intensity has been linked with positive growth indices, the use of high light intensity may potentially increase the occurrence of malformation, complicating the role light intensity plays in *S. lalandi* larviculture and suggests light intensity requirements may change with age (Cobcroft, 2013; Cobcroft et al., 2004). Malformation in larval marine fish has been associated with numerous environmental and biological factors and consequently the definitive cause is often difficult to isolate (Cobcroft and Battaglene, 2009; Debruyn et al., 2007; Hamza et al., 2012; Meier et al., 2010). Given the strong relationship of *S. lalandi* larvae to their surrounding visual environment, it would not be surprising if inappropriate lighting is a contributing factor leading to malformation. Walling behaviour is linked to jaw deformity in *L. lineata* (Cobcroft and Battaglene, 2009) and is commonly observed in *S. lalandi* larvae, where larvae display incessant swimming against the tank wall (Cobcroft, 2013). It may be that the highly reflective nature of culture tanks, attract the larvae and interrupts normal larval feeding and swimming behaviours, and this phototactic attraction increases with greater light intensity (Naas et al., 1996). In order to provide the light environment necessary to fulfil larval development, in particular swimbladder inflation, and not induce jaw deformity, it may be the use of high intensity diffuse light in conjunction with a suitably coloured tank (possibly pink or tan) provides the optimum rearing environment for *S. lalandi* larvae.

While vision is the main sensory system used for feeding by *T. maccoyii* and *S. lalandi*, the feeding experiments highlighted the importance of the non-visual sensory system, particularly *for S. lalandi*. Mechanosensory

stress may be an important culture consideration for *S. lalandi* and culture practices to reduce overstimulation of the mechanoreceptors, such as low turbulence, aeration, vibration and larval densities are likely to reduce larval stress.

6.7 Ecological considerations

At the risk of venturing beyond the immediate implications of my research into the culture of both species, the results of the short-term feeding experiments also provided potential insights into larval ecology. The feeding proficiencies displayed by *T. maccoyii* and *S. lalandi* in the behavioural experiments reflected the feeding capacities required for survival in their individual ecological niches. In order for *T. maccoyii* to survive in tropical oligotrophic waters with low or patchy prey availability (Jenkins et al., 1991; Rochford, 1962), the small larvae must capitalize on every possilbe feeding opportunity, as seen in the short-term feeding experiments where larvae fed across a broad range of treatments with great success. In contrast, the larger *S. lalandi* larvae which inhabit tropical-temperate coastal surface waters characterised by cooler waters and higher prey availability (Kolkovski and Sakakura, 2004; Smith, 1987; Sumida et al., 1985) have greater reserves and feeding opportunities. It is likely that *Seriola lalandi* can be more specialised in feeding adaptations to suit a specific environment, as seen by the restriction in feeding across the tested culture parameters to those which best matched their visual requirements.

The active choice of *T. maccoyii* to position themselves low in the water column may be reflected in the wild. Job and Bellwood (2000) suggest the majority of larvae are constrained in the depth of their vertical migrations due to the limitations of the eye, however, with increasing visual ability, generally associated with development; larvae can forage in deeper waters that contain larger zooplankton and also actively choose strata that contain higher densities of prey to increase feeding opportunities. It appears that photopic sensitivity allows *T. maccoyii* to develop an early visual advantage compared to most marine fish increasing the area

available for foraging. Miyashita et al. (2001) state that the growth strategy of *T. orientalis* is to prioritise the development of morphological structures that facilitate feeding, including the eyes, mouth and fins. Given the visual ability of the larvae it appears this is also the case for *T. maccoyii*. The potential flaw in this argument is that there is little empirical evidence currently linking *T. maccoyii* larvae to deeper, low light intensity waters. Davis et al. (1990) investigated the vertical distribution of *T. maccoyii* larvae in the wild and found that in general higher numbers of larvae were found in surface tows during the day rather than at night, however, larvae were generally associated with waters above the pycnocline (≥ 48 m). Davis et al. (1990) suggest that *T. maccoyii* larvae may feed in surface waters prior to passively dropping down to lower water layers throughout the day.

The spectral sensitivities of *T. maccoyii* and *S. lalandi* determined from microspectrophotometry and displayed in feeding experiments with coloured lights, appear to reflect the spectral environment of the larvae in the wild. *Thunnus maccoyii* larvae inhabit oligotrophic waters, where light is typified by maximum light transmission in the blue spectrum and *S. lalandi* occur in near-surface waters exposed to broad spectrum of wavelengths including red light.

6.8 Constraints of the study

6.8.1 Short-term experiments

Short-term experiments allow the rapid screening of multiple parameters providing statistically powerful information, although the experiments have three main limitations:

1. They provide only a “snap shot” of the larvae response and results must be interpreted cautiously.
2. Larvae may experience transfer stress/mortality and this may distort their responses.

3. The small size of the vessel. As the visual field of larval fish is generally one body length, it could be argued that this effect would be relatively minor.

Both species exhibited complications associated with transfer. Surface mortality was observed in *T. maccoyii* and a poor feeding response, possibly associated with stress, was observed in *S. lalandi*. The overall mortality in *T. maccoyii* was high and it was possible that surviving larvae may not have behaved normally due to the stress of transfer. However, this did not appear to be the case, as supported by three observations. First, the feeding response of the surviving larvae appeared unaffected as reflected in the high percentage of larvae feeding across all experiments. Second, the addition of oil significantly reduced mortality in *T. maccoyii* with no difference observed in the proportion or intensity of feeding, confirming that the surviving larvae were not compromised in their feeding capacity (Chapter 3). Finally, the average feeding rate in the larviculture source tanks were similar to the experimental system (Chapters 2 and 3). In comparison, while *S. lalandi* did not experience transfer mortality, it appeared that *S. lalandi* were more affected by transfer stress, took longer to recover, and did not display the same capacity to initiate feeding as *T. maccoyii*. While a proportion of the *S. lalandi* cohorts ingested food on 2 dph, it is well documented that first-feeding occurs on 3 dph (Carton, 2005; Chen et al., 2006; Ma and Qin, 2012). Behavioural experiments were initiated on 3 dph for both species as the larvae possessed fully pigmented eyes and had fully consumed their endogenous yolk indicating the need to commence feeding, which agrees with studies reported by Woolley et al. (2009) and Chen et al. (2006). The results based on first-feeding *T. maccoyii* and the second day of feeding for some *S. lalandi* larvae had the potential to confound the experiments as a number of *S. lalandi* larvae may have had prior experience feeding possibly increasing the likelihood of feeding success. This does not appear to be the case as comparative feeding rates were observed by Carton (2005) in naïve *S. lalandi* first-feeding 3 dph larvae and my study.

While the short-term experiments have constraints, this experimental approach is novel and allows screening of factors to optimise culture at specific larval developmental stages. In addition, the possible interaction of environmental factors can be identified rapidly and at a relatively low cost (Nicolaisen et al., 2013).

6.8.2 Microspectrophotometry

Larvae were collected from the Clean Seas Tuna Ltd hatchery at Arno Bay, South Australia. Due to the remote location, microspectrophotometry could not be conducted on fresh samples, so samples were frozen and shipped to Perth in Western Australia. To my knowledge this is the first time that microspectrophotometry samples have been collected from larval fish of any species and frozen for later analysis. This allowed the acquisition of valuable MSP data. While spectral data was limited, and therefore should be interpreted cautiously, it does provide an indication of the visual sensitivities of both species.

6.8.3 Relevance to wild ecology

As a final caveat and constraint, care needs to be taken in extrapolating the results of larval culture experiments to help elucidate the ecology of wild larvae, especially as the nature of light in the ocean behaves very differently to that in a small land-based container under artificial light (Naas et al., 1996).

6.9 Factors requiring testing

My study has raised a number of questions that warrant further investigation. For *T. maccoyii*:

1. The effect of long-term larviculture under low light intensity conditions, addressing the specific question of whether surface mortality and sinking mortality could be reduced at low light intensity.
2. The effect of long-term larviculture under blue light.

3. Investigation into the absolute sensitivities to determine the threshold where cone-mediated vision takes over from rod-mediated vision.

and for *S. lalandi*:

1. The effect of long-term culture in pink or tan tanks.
2. The effect of long-term rearing under red lights.
3. Further investigation into the effect larval density has on the disturbance of larval feeding.
4. Examination of the effect of high intensity, diffuse light on walling behaviour, feeding and swimbladder inflation.
5. Investigation into the absolute sensitivities to determine the threshold where cone-mediated vision takes over from rod-mediated vision.

6.10 Conclusion

In the pursuit of investigating the comparative visual performance of larval *T. maccoyii* and *S. lalandi*, my study revealed a number of retinal adaptations and behavioural responses that contribute to our knowledge of feeding capacity and give indications of environmental variables that are conducive and deleterious to larval survival. The light environment is of paramount importance to *T. maccoyii* and *S. lalandi* larvae, although both species exhibit very different light requirements and visual capabilities. This has important culture implications. *Thunnus maccoyii* display morphometric and behavioural adaptations to life and feeding in low light environments. Evidence of this includes:

- Linear cell density, angular cell density and MSA specialisation in the ventral retinal region suggesting the larvae are visually equipped to feed and avoid predators in the dorsal visual plane i.e., from above them.

- High early photopic sensitivity allowing increased foraging in lower light intensity environments.

- High mortality during early larval history when exposed to high light conditions in culture.
- Early development of the retinomotor response most likely associated with protection of larval eyes from high light environments.
- High relative photon capture area to ganglion cell increasing photopic acuity and sensitivity increasing the capacity for maximum photon capture and visual information processing received from dorsally captured low light conditions, most likely associated with deeper waters.
- Visual pigment sensitivity in the blue region allowing greater feeding at depth and during dawn and dusk.

In contrast, *S. lalandi* larvae display morphometric and behavioural adaptations to life in high light environments. Evidence of this includes:

- Linear cell density and angular cell density specialisation in the dorsal retinal region suggesting the larvae are associated with surface waters and are visually equipped to feed and avoid predators in the ventral visual plane i.e., below them.
- Longer light path in the dorsal retinal region.
- Increased feeding under high light conditions.
- Improved feeding under red spectrum light most likely associated with near-surface water conditions.
- Visual pigment sensitivity in the green spectrum associated with well-lit coastal waters.

The major culture implications taken from my study in order to produce quality seed stock include:

1. *Thunnus maccoyii* appear to require a low light environment, particularly during the first two weeks of culture prior to the development of RPE migration.
2. The culture environment has profound effects on first-feeding *S. lalandi* and requires strict control to ensure optimum conditions are presented to the larvae. *Seriola lalandi* display an ongoing need for high light intensity conditions throughout culture.

My study revealed fundamental differences in the ontogeny and capacity of the visual system in *T. maccoyii* and *S. lalandi*. While both species were able to feed within a few days of hatching, and this ability increased with age, the feeding response of the larvae to the visual environment was notably different. Species-specific retinal adaptations were identified, highlighting differences between the visual systems of *T. maccoyii* and *S. lalandi*, which help explain the observed feeding performance of the larvae. The improved understanding of visual capacity in larvae of both species gained from my study provides important information on the culture requirements of the larvae and insight into the larval ecology in the wild.

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Appendices

Retinal counts and statistical summaries

The following tables represent the average retinal cell densities, angular cell densities and statistical summaries of cell density, angular density and cell thickness layers in *T. maccoyii* and *S. lalandi*.

Appendix 1. *Thunnus maccoyii* and *Seriola lalandi* retinal region cell densities per 100 µm transect averaged over time (3 dph to 30 dph).

Retina cell	*Thunnus maccoyii* (µm)	*Seriola lalandi* (µm)
Cone	Dorsal = 38.82 ± 5.48	Dorsal = 41.00 ± 6.31
	Medial = 39.58 ± 5.98	Medial = 40.38 ± 5.32
	Ventral = 45.94 ± 6.12	Ventral = 38.71 ± 4.67
Rod	Dorsal = 57.24 ± 30.92*	Dorsal = 51.00 ± 19.58
	Medial = 36.86 ± 23.34*	Medial = 32.86 ± 18.34
	Ventral = 50.50 ± 35.02*	Ventral = 38.50 ± 11.08
All nuclei	Dorsal = 58.24 ± 24.84*	Dorsal = 58.29 ± 28.04*
	Medial = 52.04 ± 18.56*	Medial = 50.38 ± 16.66*
	Ventral = 64.78 ± 28.56*	Ventral = 52.75 ± 18.02*
Horizontal	Dorsal = 12.12 ± 4.80	Dorsal = 10.88 ± 2.92
	Medial = 11.74 ± 3.14	Medial = 12.38 ± 3.58
	Ventral = 16.20 ± 4.76	Ventral = 13.17 ± 3.07
Bipolar	Dorsal = 133.50 ± 26.64*	Dorsal = 132.21 ± 19.59*
	Medial = 98.24 ± 21.50*	Medial = 111.38 ± 23.86*
	Ventral = 183.58 ± 37.56*	Ventral = 140.13 ± 25.79*
Ganglion	Dorsal = 73.12 ± 33.54*	Dorsal = 46.79 ± 23.60*
	Medial = 48.33 ± 25.69*	Medial = 38.67 ± 22.33*
	Ventral = 67.77 ± 25.54*	Ventral = 47.96 ± 24.91*

N.B. * denotes a significant interaction has been shown between retinal region and increasing age.

Appendix 2. Statistical summary of *Thunnus maccoyii* and *Seriola lalandi* linear cell densities as affected by retinal region, age and interaction between retinal region and age.

Retinal cell	Factor	Significance	
		Thunnus maccoyii	*Seriola lalandi*
Cone	Region	$F_{2, 54} = 15.111$, $P < 0.001$	$F_{2, 54} = 1.484$, $P = 0.236$
	Age	$F_{5, 54} = 8.027$, $P < 0.001$	$F_{5, 54} = 3.670$, $P = 0.006$
	Interaction	$F_{10, 54} = 0.638$, $P = 0.774$	$F_{10, 54} = 1.889$, $P = 0.067$
Rod	Region	$F_{2, 12} = 18.191$, $P < 0.001$	$F_{2, 54} = 10.470$, $P = 0.001$
	Age	$F_{3, 12} = 114.29$, $P < 0.001$	$F_{5, 54} = 106.48$, $P < 0.001$
	Interaction	$F_{6, 12} = 8.064$, $P = 0.001$	$F_{10, 54} = 2.123$, $P = 0.149$
Ganglion	Region	$F_{2, 54} = 66.964$, $P < 0.001$	$F_{2, 54} = 8.914$, $P < 0.001$
	Age	$F_{5, 54} = 32.322$, $P < 0.001$	$F_{5, 54} = 108.14$, $P < 0.001$
	Interaction	$F_{10, 54} = 6.376$, $P < 0.001$	$F_{10, 54} = 2.511$, $P = 0.015$
Bipolar	Region	$F_{2, 54} = 66.922$, $P < 0.001$	$F_{2, 54} = 5.447$, $P < 0.001$
	Age	$F_{5, 54} = 0.0871$, $P = 0.507$	$F_{5, 54} = 21.072$, $P < 0.001$
	Interaction	$F_{10, 54} = 2.584$, $P = 0.012$	$F_{10, 54} = 6.698$, $P = 0.015$
Horizontal	Region	$F_{2, 54} = 12{,}202$, $P < 0.001$	$F_{2, 54} = 4.713$, $P = 0.013$
	Age	$F_{5, 54} = 7.676$, $P < 0.001$	$F_{5, 54} = 7.904$, $P < 0.001$
	Interaction	$F_{10, 54} = 1.446$, $P = 0.186$	$F_{10, 54} = 0.821$, $P = 0.610$
Photoreceptor nuclei	Region	$F_{2, 54} = 14.132$, $P < 0.001$	$F_{2, 54} = 8.613$, $P = 0.001$
	Age	$F_{5, 54} = 104.60$, $P < 0.001$	$F_{5, 54} = 119.37$, $P < 0.001$
	Interaction	$F_{10, 54} = 2.124$, $P = 0.038$	$F_{10, 54} = 4.369$, $P < 0.001$

Appendix 3. The angular cell density of *Thunnus maccoyii* and *Seriola lalandi* defined by retinal cell type and larval age.

Retinal cell	Age (days post-hatching)	*T. maccoyii* (10' visual arc)	*S. lalandi* (10' visual arc)
Cones	3	0.16 ± 0.01	0.15 ± 0.02
	9	0.16 ± 0.01	0.21 ± 0.02
	12	0.26 ± 0.04	0.22 ± 0.02
	15	0.24 ± 0.03	0.26 ± 0.03
	21	0.54 ± 0.11	0.56 ± 0.08
	30	1.13 ± 0.28	0.97 ± 0.20
Rods	21	0.29 ± 0.13	0.85 ± 0.56
	30	2.52 ± 1.04	0.96 ± 0.63
Photoreceptor nuclei	3	0.16 ± 0.01	0.15 ± 0.01
	9	0.16 ± 0.02	0.21 ± 0.02
	12	0.28 ± 0.06	0.22 ± 0.02
	15	0.24 ± 0.02	0.26 ± 0.04
	21	0.83 ± 0.19	0.95 ± 0.18
	30	3.66 ± 1.25	2.40 ± 0.40
Ganglion cells	3	0.15 ± 0.01	0.06 ± 0.08
	9	0.10 ± 0.02	0.13 ± 0.07
	12	0.22 ± 0.05	0.17 ± 0.04
	15	0.20 ± 0.07	0.15 ±0.05
	21	0.45 ± 0.22	0.25 ± 0.09
	30	0.82 ±0.28	0.32 ± 0.07

Appendix 4. Statistical summary of *Thunnus maccoyii* and *Seriola lalandi* angular cell densities as affected by retinal region, age and interaction between retinal region and age.

Retinal cell	Factor	Significance	
		T. maccoyii	*S. lalandi*
Cones	Region	$F_{2, 54} = 7.316$, $P = 0.002$	$F_{2, 54} = 1.736$, $P = 0.186$
	Age	$F_{5, 54} = 222.07$, $P < 0.001$	$F_{5, 54} = 211.512$, $P < 0.001$
	Interaction	$F_{10, 54} = 1.221$, $P = 0.299$	$F_{10, 54} = 0.984$, $P = 0.468$
Rods	Region	$F_{2, 18} = 2.119$, $P = 0.149$	$F_{2, 54} = 10.699$, $P = 0.001$
	Age	$F_{1, 18} = 115$, $P < 0.001$	$F_{5, 54} = 224.046$, $P < 0.001$
	Interaction	$F_{2, 18} = 8.064$, $P = 0.001$	$F_{10, 54} = 3.344$, $P = 0.058$
Ganglion cells	Region	$F_{2, 54} = 1.098$, $P = 0.341$	$F_{2, 54} = 14.809$, $P < 0.001$
	Age	$F_{5, 54} = 63.193$, $P < 0.001$	$F_{5, 54} = 28.012$, $P < 0.001$
	Interaction	$F_{10, 54} = 2.036$, $P = 0.047$	$F_{10, 54} = 1.688$, $P = 0.108$
Photoreceptor nuclei	Region	$F_{2, 54} = 2.841$, $P = 0.067$	$F_{2, 54} = 9.179$, $P < 0.001$
	Age	$F_{5, 54} = 218.00$, $P < 0.001$	$F_{5, 54} = 499.145$, $P < 0.001$
	Interaction	$F_{10, 54} = 0.927$, $P = 0.516$	$F_{10, 54} = 4.510$, $P < 0.001$

Appendix 5. Statistical summary of cell layer thickness measured in *Thunnus maccoyii* and *Seriola lalandi* in the ganglion cell layer (GCL), inner nuclear layer (INL), inner plexiform layer (IPL), outer nuclear layer (ONL) and the pigment epithelium and outer segments i.e. light path (PE + OS). The retinal regions are abbreviated as dorsal (D), medial (M) and ventral (V).

Retinal region	Two-way ANOVA: age and area	Observation
T. maccoyii GCL	Interaction $F_{10, 54} = 3.755$, $P = 0.001$	General increase with age and V generally greater
T. maccoyii INL	Age $F_{5, 54} = 9.967$, $P < 0.001$ Area $F_{2, 54} = 35.584$, $P < 0.001$	General increase with age M < V and D
T. maccoyii IPL	Interaction $F_{10, 54} = 2.503$, $P = 0.015$	General increase with age and V generally greater
T. maccoyii ONL	Age $F_{5, 54} = 14.028$, $P < 0.001$	Increase with age
T. maccoyii PE + OS	Interaction $F_{10, 54} = 3.331$, $P = 0.002$	General increase with age and V and D > M at 30 dph
S. lalandi GCL	Interaction $F_{10, 54} = 2.068$, $P = 0.044$	No observed trend
S. lalandi INL	Interaction $F_{10, 54} = 2.267$, $P = 0.027$	General increase with age
S. lalandi IPL	Age $F_{5, 54} = 12.001$, $P < 0.001$ Area $F_{2, 54} = 8.483$, $P < 0.001$	General increase with age M < V and D
S. lalandi ONL	Interaction $F_{10, 54} = 2.387$, $P = 0.020$	General increase with age and D > at 30 dph.
S. lalandi PE + OS	Interaction $F_{10, 54} = 3.564$, $P = 0.001$	General increase with age and D > at 21 and 30 dph