Aspects of the morphological development and feeding performance of larval striped trumpeter (*Latris lineata*) in culture

by

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Declarations

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, and to the best of my knowledge and belief is original material, containing no material previously published or written by another person except where due acknowledgment is made in the text of the thesis.

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Abstract

Striped trumpeter (*Latris lineata*) is a new candidate species for aquaculture in temperate Australia. Survival of larvae prior to flexion has proven a bottleneck in the production of this species for culture. In addition, almost all juveniles cultured to date exhibit malformations of the jaw that may impede larval feeding success. Body size and morphology impose constraints upon feeding success in larvae of broadcast spawning fishes. Furthermore, larvae have an absolute reliance upon sense organs for the detection and subsequent capture of prey. In this study, aspects of morphological development and feeding performance were described in larval striped trumpeter. Chemosensory and mechanosensory organs were present and presumed functional soon after hatching, while the eye was functional coincident with first-feeding on day 7 post-hatching. The structure of the photoreceptors in different regions of the retina of the larvae suggested the area specialised for the most acute image formation corresponded to a visual field in the fronto-ventral region. Analysis of videocinematography of feeding larvae in the horizontal plane confirmed a forward-directed functional visual field. The area of the visual field increased with larval ontogeny from day 13 to day 17 post-hatching, due to the wider range of reactive angles used by older larvae. Maximum reactive distances of larvae to rotifer prey (~5.1mm) were 97% of larval standard length, while the distance at which larvae initiated a strike at the prey was much lower (~0.45mm) at 8% of larval standard length. Visual angles determined from larval feeding behaviour were higher than the minimum separable angles predicted by histology, such that the functional acuity of the larvae was not as good as that predicted by retinal structure. Jaw malformation was only evident in post-flexion larvae greater than 10 mm standard length and was characterised by an open jaw in which cartilage and bone elements appeared structurally normal but were in abnormal positions. The effects of light intensity and microalgal cell density (turbidity) on larval feeding behaviour were assessed in short-term feeding trials. None of the pre-flexion larvae used to investigate optimal light conditions for feeding exhibited jaw malformations. Larvae fed equally well in clearwater (no microalgal cells present) in a light intensity range of 1-10 $\mu mol.s^{-1}.m^{-2}$. An ontogenetic

improvement in photopic visual sensitivity of larvae was indicated by improved feeding at 0.1 $\mu mol.s^{-1}.m^{-2}$ between day 8 and day 23 post-hatching. Algae-induced turbidity had different effects on larval feeding response dependent upon the previous visual environment of the larvae. Young larvae, day 9 post-hatching, reared in clearwater showed decreased feeding capabilities with increasing turbidity, while older clearwater reared larvae fed well at all turbidities tested. Likewise, greenwater (with microalgal cells present) reared larvae had increased feeding capabilities in the highest algal cell densities tested compared with those in low algal cell density, and clearwater to which they were naive. This study demonstrated that striped trumpeter larvae are primarily visual feeders with a small visual field relative to larval body size, that jaw malformation is unlikely to impede feeding in pre-flexion larvae, and that greenwater may provide a benefit to larval feeding although the previous visual environment of larvae affected subsequent feeding responses.

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Figure 5.2. *Camera lucida* drawings of the lateral view of cartilages (stippled) and bones (non-stippled) of the cranial skeleton of cleared and stained cultured striped trumpeter larvae, normal development on days 17 (a), 22 (b), 30 (c), and 44 (d), and jaw malformation evident on days 44 (e), 48 (f), and 62 (g) post-hatching, and ventral view of the anterior lower jaw (h) of day 62 larva drawn in (g). Sclerotic is omitted from b-g. Abbreviations: An, angular; Ac, asymmetric cartilage; aTr, anterior remnants of trabecula; Auc, auditory capsule; Bas, basisphenoid; Bo, Basioccipital; Br, branchiostegal ray; Ca, extraneous cartilage ventral to symphysial joint; Ch, ceratohyal; Ch-Eh, ceratohyal-epihyal cartilage; De, dentary; Ecp, ectopterygoid; Eeb, ectethmoid bar; Eh, epihyal; Enp, endopterygoid; Et, epiphysial tectum; Etp, ethmoid plate; Ex, exoccipital; Fr, frontal; Gh, glossohyal; gSyj, gap at symphysial joint; Hh, hypohyal; Hm, hyomandibular; Hm-Sy, hyomandibular-symplectic cartilage; Ih, interhyal; Le, lateral ethmoid; Ma, maxilla; Mc, Meckel's cartilage; Mp, Meckel's dorsal process; Mt, medial tectum (*taenia tecta medialis*); Oc, olfactory capsule; Pal, palatine; Pc, parachordal cartilage; Pm, premaxilla; Ps, parasphenoid; pTr, posterior remnants of trabecula; Qp, quadrate posterior process; Qu, quadrate; Ra, retroarticular; Rc, rostral cartilage; Scl, sclerotic; Sob, supraorbital; Sy, symplectic; Tr, trabecula; Vo, vomer. Scale bars are 200 μm. Larvae drawn are representative of each age, in which mean SL ± SE and sample number (n) were a) 6.9 ± 0.1 mm (n = 7), b) 7.5 ± 0.1 mm (n = 5), c) 7.9 ± 0.5 mm (n = 5), d) 9.3 mm, e) 11.2 ± 0.8 mm (n = 3), f) 11.4 ± 0.6 mm (n = 10), and g) & h) 15.1 ± 1.3 mm (n = 2). 144

Figure 5.3. X-radiographs of the lateral view of the cranial skeleton of striped trumpeter. a) a normal wild caught adult (47.0 cm FL, 2105 g), b) a relatively normal cultured juvenile (31.0 cm FL, 470 g), c) a malformed cultured juvenile (41.5 cm FL, 950 g), and d) a malformed cultured juvenile (fixed head with eyes removed) (32.0 cm FL, 390 g). Abbreviations: An, angular; bEtp, ethmoid plate bent ventrally; De, dentary; eMa, enlarged maxilla; Gh, glossohyal; gSyj, abnormal gap between adjacent dentary bones at the symphysial joint; Pm, premaxilla; Syj, symphysial joint. Scale bar is 2 cm. 147

Figure 5.4. X-radiographs of the dorsal (a-d) and frontal (e-h) view of the anterior tip of the lower jaw dissected from four malformed, cultured striped trumpeter juveniles. Abbreviations: An, angular; dDe, dentary with abnormal increase in dorso-ventral depth; De, dentary; gSyj, abnormal gap between adjacent dentary bones at the symphysial joint; Syj, symphysial joint. Scale bar is 2 cm. 148

Figure A2.1. Spectral irradiance from a range of light sources measured with a Li-Cor LI-1800 portable spectroradiometer. 186

1. Chapter One. General Introduction

1.1 Larvae of broadcast spawning marine teleosts

Broadcast spawning marine teleosts are generally highly fecund, producing thousands of small eggs (Bromage, 1995). Their larvae hatch at a small size and early stage in development (Blaxter, 1988). The growth and survival of their offspring is dependent upon several factors, including the ability to avoid predators and the ability to feed (Blaxter, 1986). The development of larval organ systems required for feeding is rapid as endogenous yolk reserves are quickly utilised and exogenous nutrition is required (Blaxter, 1988). Feeding behaviour of marine teleost larvae involves a complex interaction between larval capabilities and external environmental factors, such as prey density and distribution, light intensity, turbidity, and turbulence (Hunter, 1980; Blaxter, 1986; Chesney, 1989). Fish larvae are absolutely reliant upon sensory organs, developed jaw structures, musculature suited to swimming and orientation, and sufficient gut development for the assimilation of food (Blaxter, 1988). They are generally highly selective planktivores, and most are primarily visual feeders, thus requiring appropriate light conditions for feeding (Blaxter, 1986; Huse, 1994).

Many species of broadcast spawning teleosts are cultured commercially throughout the world, and others are under investigation as potential candidates for culture (Bromage, 1995). The examination of the morphological development of each species is important for understanding the requirements of larvae in culture situations (Blaxter, 1988; Howell *et al.*, 1998; Boglione *et al.*, 2001). In addition, behavioural observations are important in developing appropriate culture techniques (Hunter, 1980; Brown *et al.*, 1997). Together, morphological and behavioural observations improve the information base regarding larval biology generally, and particularly advance the knowledge of the species studied, thus providing additional information if problems arise

during culture, and removing the trial and error nature of determining rearing methods.

1.2 Striped trumpeter - general information

Striped trumpeter (*Latris lineata*) naturally occur in temperate south-eastern waters of Australia (Fig.1.1), extending from Tasmania to eastern South Australia, Victoria and to southern New South Wales, and are also present in New Zealand and South American waters (Last *et al.*, 1983; Kailola *et al.*, 1993). Adults (Fig.1.2) are associated with offshore rocky bottom between 50 and 300m depth, while juveniles (up to 2kg) were once common in shallow coastal water but are now usually found in more remote areas (Last *et al.*, 1983). The adults and juveniles feed on large invertebrates, and crustacean zooplankton (Kuiter, 1993). They grow to at least 1.2m total length and 25kg (Last *et al.*, 1983). Striped trumpeter adults spawn from late winter to early spring (August to October) (Kailola *et al.*, 1993). However, very little is known of the biology of the egg and larval stages in the wild, until the juveniles move to shallow inshore reefs around January at approximately 18cm fork length and likely more

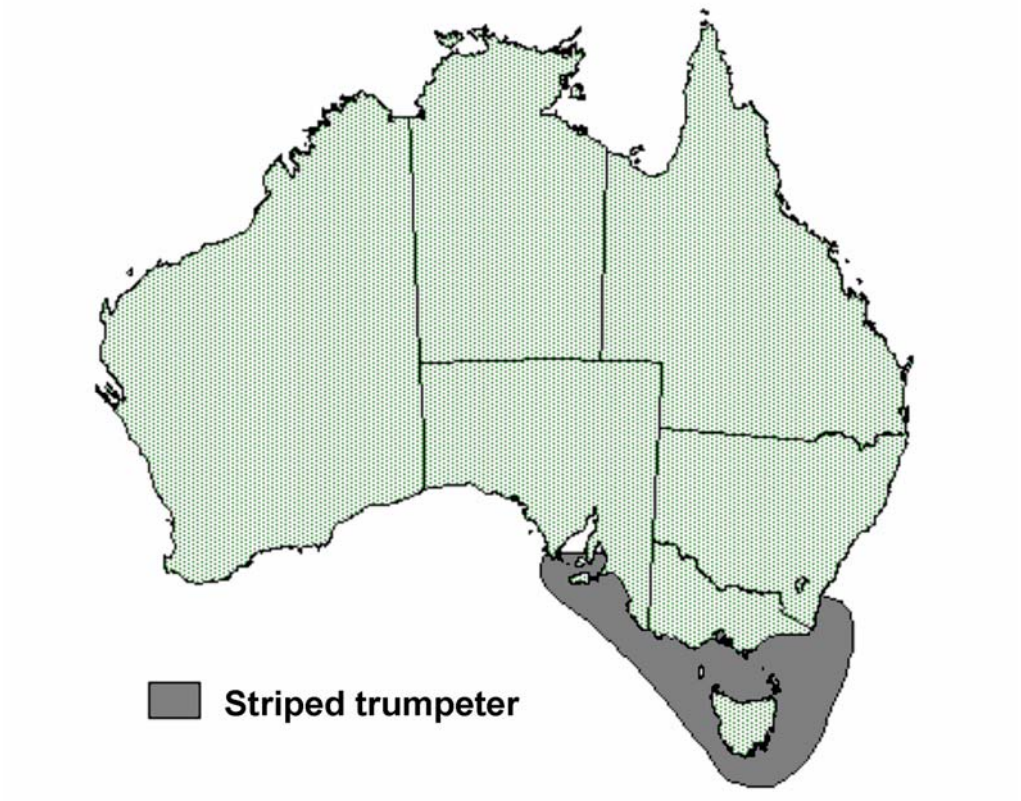

Figure 1.1. The approximate distribution of striped trumpeter (*Latris lineata*) in south-eastern Australian waters. (Map provided by Dr D. Morehead.)

than 12 months of age (Lyle and Hodgson, 2001). The larvae of striped trumpeter have been described, largely from cultured specimens (Furlani, 1998; Furlani and Ruwald, 1999), although a small number of larvae (<10mm standard length, SL) have been collected from the wild in September/October at nearshore (30-50m depth) and shelf-edge (~200m depth) sites on the west coast of Tasmania (Furlani and Ruwald, 1999). The larvae are similar in appearance to other species that have a highly protracted post-larval "paperfish" stage (e.g. Jackass morwong *Nemadactylus macropterus*) in which they are laterally compressed, with dorso-ventrally deep silvery bodies (Bruce, 1998; Furlani and Ruwald, 1999). Paperfish apparently dwell at the surface of the water column (neustonic), and are mainly caught offshore (Furlani and Ruwald, 1999). Further sampling is required to confirm such a distribution in striped trumpeter (Furlani and Ruwald, 1999). Given their morphological similarity to other paperfish, the timing of their settlement on reefs, and the development of reared fish, metamorphosis from the paperfish stage to the juvenile probably occurs about nine months from hatching (Morehead *et al.*, 1999; Lyle and Hodgson, 2001), and at around 25g in reared fish (Morehead, unpublished data).

According to Last *et al.* (1983), striped trumpeter are "highly esteemed as a food fish and must rank amongst the finest in Australian waters". There has been an increase in the reported catch of the commercial fishery (Tasmania and Commonwealth) for this species from 75 tonnes in 1990/1991 to 101 tonnes in

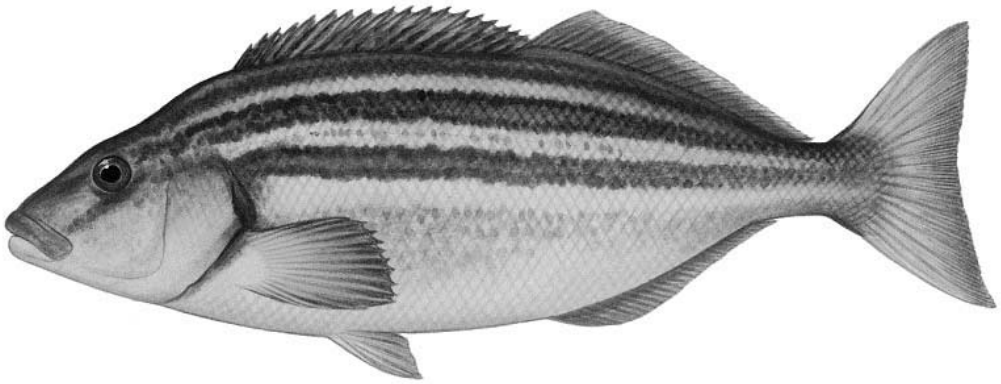

Figure 1.2. Illustration of an adult striped trumpeter (*Latris lineata*). (Illustration provided by the Tasmanian Department of Primary Industries, Water and Environment.)

1999/2000, ranging from 53 to 108 tonnes in that period (Lyle and Hodgson, 2001). Adult fish are caught by hook fishing methods in depths of 40-160m and juveniles are predominantly taken with graball gillnets usually in depths less than 20m (Lyle and Hodgson, 2001). Striped trumpeter is also an important species to recreational fishing in Tasmania. The amount of striped trumpeter caught by the recreational gillnet fishery was almost 80% of that caught by the commercial gillnet fishery from December 1996 to April 1998 (Lyle and Hodgson, 2001).

The striped trumpeter is a candidate for temperate marine aquaculture, due to its high quality white flesh, which is highly suitable for the Japanese sashimi market (Ruwald, 1991; Searle and Zacharin, 1994). Aspects of the biology of striped trumpeter and rearing techniques have been under investigation at the Tasmanian Aquaculture and Fisheries Institute, Marine Research Laboratories in Taroona (formerly Tasmanian Department of Primary Industry and Fisheries facility) for 12 years. Techniques for managing wild-caught broodstock of this species and producing good quality eggs are well-established (Hutchinson, 1994; Morehead, 1998; Morehead *et al.*, 1998; Morehead *et al.*, 2000; Morehead *et al.*, 2001). The broodstock readily acclimate to captivity in a tank situation and to chopped fish or manufactured diets, and may be handled regularly with minimal impact on egg production (Morehead *et al.*, 2000). In addition, naturally spawned fertilised eggs may be collected from the broodstock holding tanks (Morehead *et al.*, 2000). Striped trumpeter are multiple spawners, producing thousands of small eggs (1.2-1.4mm diameter) each season (Morehead *et al.*, 1998; Morehead *et al.*, 2000). Semen can be successfully cryopreserved and used to fertilise eggs, providing the opportunity to store genetic material (Ritar, 1999). The temperature for egg incubation has been investigated (Bermudes and Ritar, 1999; Morehead *et al.*, submitted) and the morphological development of cultured larvae has been described (Furlani, 1991). However, larviculture has proved the bottleneck to production due to low larval survival (Ruwald *et al.*, 1991; Pankhurst and Hilder, 1998), and malformations of the swimbladder and jaw are evident in surviving fish (Morehead *et al.*, 1999; Cobcroft *et al.*, 2001; Trotter *et al.*, 2001).

1.3 Striped trumpeter - larvae

Like many broadcast spawning marine teleosts, striped trumpeter hatch at an early stage in development. Larvae hatch 6 to 8 days post-fertilisation and at 3.2 to 3.6mm total length, depending on temperature of incubation (Ruwald *et al.*, 1991; Bermudes and Ritar, 1999; Furlani and Ruwald, 1999) (Fig.1.3a). At hatching, the eyes are not pigmented, the mouth and jaw apparatus are not formed, the gut is a simple straight tube, and they have a large yolk and a single oil drop as endogenous energy reserves until they start to feed (Ruwald *et al.*, 1991; Furlani and Ruwald, 1999) (Fig.1.3b). The timing of first-feeding is temperature dependent and occurs around 10 days post-hatching (~5.1mm SL) at 13 ± 2°C (Ruwald *et al.*, 1991), and around 7 days post-hatching at 14°C (Pankhurst and Hilder, 1998) (Fig.1.3c). There has been limited success in rearing striped trumpeter larvae; small numbers of juveniles were produced in 1992 at the University of Tasmania at Launceston Key Centre for Aquaculture (Hutchinson, 1994), in 1994, 1997 and three consecutive batches in 1999 at the

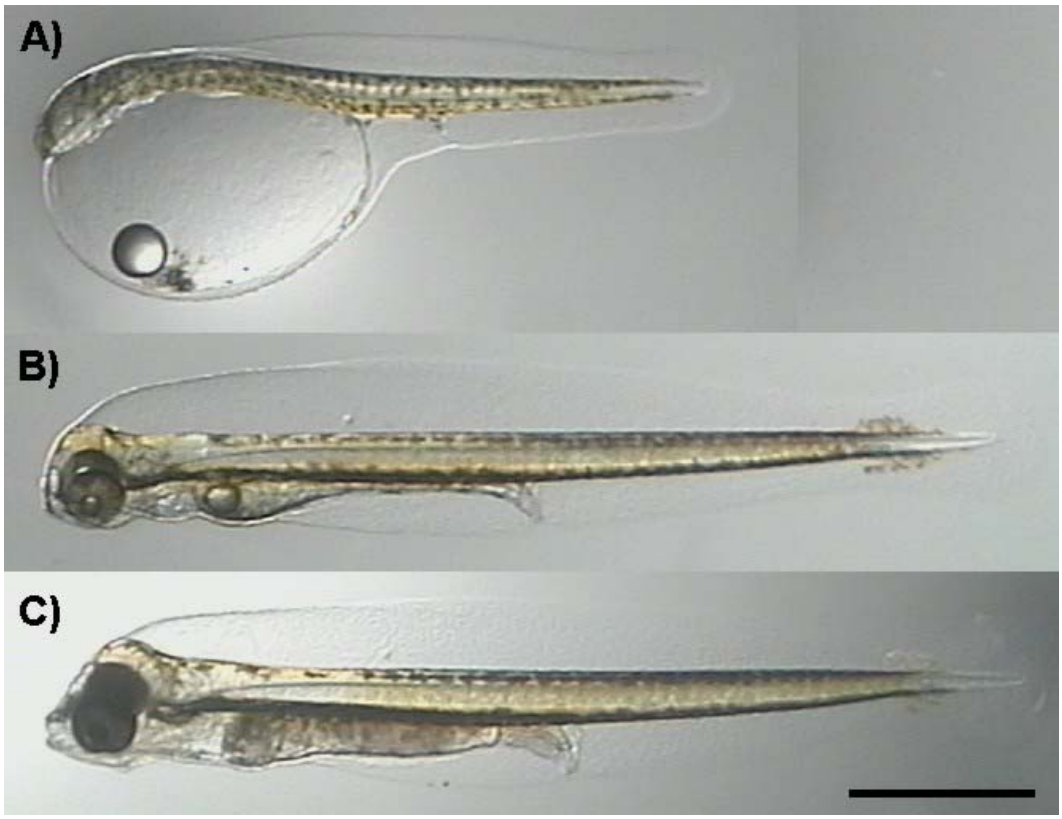

Figure 1.3. Striped trumpeter yolksac larvae at hatching, day 0 (A), and on day 5 (B), and feeding larva on day 8 (C) post-hatching. Scale bar is 1mm.

Marine Research Laboratories in Taroona (Morehead *et al.*, 1999). In general, survival is good through egg and yolksac larvae incubation periods, with the bottleneck in production occurring between first-feeding and metamorphosis to the paperfish stage. Larvae complete metamorphosis to paperfish at approximately 10-15mm SL and 40-50 days post-hatching (Cobcroft personal observ.).

1.4 Scope and objectives

As part of a broader project within the Co-operative Research Centre for Aquaculture investigating the development of production techniques for striped trumpeter, this study focussed on the morphological development of the larvae in relation to their feeding behaviour. Striped trumpeter larvae were observed to display aberrant behaviour in earlier rearing trials, including avoiding open water and swimming with their heads against the walls of the tank, and had poor feeding performance at high light intensity (Pankhurst and Hilder, 1998). These are symptoms that have been attributed to inappropriate light conditions in culture (Huse, 1994; Rieger and Summerfelt, 1997). It was subsequently demonstrated that the retinal structure of striped trumpeter larvae indicates they are equipped for visual feeding (Pankhurst and Hilder, 1998). In a preliminary, unreplicated experiment, Pankhurst and Hilder (1998) found that striped trumpeter were primarily visual feeders and displayed an increase in visual sensitivity with age, such that the proportion of larvae feeding increased with age under low light (1 lux, ~ 0.01 $\mu mol.s^{-1}.m^{-2}$) and decreased under high light intensity (700 lux, ~ 8.4 $\mu mol.s^{-1}.m^{-2}$). Pankhurst and Hilder (1998) demonstrated that the light environment for optimal feeding changes with ontogeny in larval striped trumpeter.

Striped trumpeter, like most other teleost larvae, are apparently primarily visual feeders. However, prior to the present study, the information on larval feeding in culture was limited. Nothing was known of the development of the non-visual sensory organs of striped trumpeter, nor the relative contribution of different senses to larval feeding behaviour. The effect of light intensity on larval feeding

performance remained to be tested in replicated trials. The use of microalgae in larval culture (known as greenwater) had promising results in the rearing of striped trumpeter (Ruwald *et al.*, 1991), although the effect of greenwater on feeding has not been assessed. In addition, the impact of the jaw malformation visible in post-flexion fish, on the feeding performance of pre-flexion larvae was unknown. The present study investigated all of these aspects of larval development and feeding in order to optimise feeding success of striped trumpeter larvae in culture. The development of two systems vital for successful feeding in cultured striped trumpeter, the sensory organs and the jaw, were described. In addition, the sensory basis of feeding was examined, and the appropriate light conditions for feeding were determined.

1.4.1 Constraints on the present study

The initial intent of this project was to examine the development and feeding performance of larvae from hatching to metamorphosis. However, variability in the survival, growth, and health of reared larvae limited the availability of larvae, particularly older larvae, for observations. Sensory organ development was assessed in larvae to day 26 post-hatching, and feeding performance was examined in larvae up to day 23 post-hatching. Jaw development and malformation were described in larvae and post-flexion larvae up to day 62 post-hatching.

1.4.2 Sensory organs

Vision, a light dependent sensory function, is considered to be the primary sense involved in the feeding behaviour of small marine finfish larvae (Blaxter, 1986), and preliminary results indicate this is the case in striped trumpeter larvae (Pankhurst and Hilder, 1998). However, recent studies examining feeding behaviour in the dark have shown that non-visual senses also play a role in the feeding behaviour of some fish larvae (Salgado and Hoyt, 1996) and juveniles (Batty and Hoyt, 1995). As small marine larvae grow and their sensory organs develop, the constraints imposed on them at hatch by small body size and early

stage of development are overcome, reflected in changes in feeding abilities (Blaxter, 1986). Fish larvae are absolutely reliant upon sensory organs for the detection of prey. Understanding the dynamics of sensory functional capabilities is an important requirement in optimising conditions for prey capture by fish larvae in intensive culture conditions. Since samples of striped trumpeter larvae from the wild are limited, cultured animals were relied upon for the examination of sensory organ development and behavioural function to develop appropriate culture conditions for this species. Sensory organ development in striped trumpeter was also compared with that of other species, enabling the prediction of the ecology of the larvae in the wild.

Objective: Describe the development of sensory organs in larval striped trumpeter.

Objective: Determine the contribution of non-visual sensory organs to feeding behaviour in larval striped trumpeter.

1.4.3 Feeding Behaviour

Behavioural studies have shown that the distances at which teleost larvae react to planktonic prey (reactive distance) are often small, in the order of a body length (Blaxter, 1986). Given the limited visual field of larvae, it is important to establish the functional capabilities of larvae in order to provide them with adequate conditions for feeding in culture (e.g. prey density). The functional visual field of feeding striped trumpeter larvae was previously unknown. Histological assessment of different regions of the retinae of fishes has enabled the prediction of their visual capabilities, particularly acuity (Williamson and Keast, 1988; Pankhurst, 1989), however these tend to be overestimates in relation to behaviourally determined capabilities (Browman *et al.*, 1990; Pankhurst *et al.*, 1993; Pankhurst, 1994). This study used videocinematography to assess the functional visual field, in the horizontal plane, of striped trumpeter larvae feeding under typical culture conditions, and compared that with the

predicted visual field determined from morphological assessment of different regions of the retina.

Objective: Describe the functional visual field of feeding larval striped trumpeter with ontogeny and compare it with the theoretical visual field determined from retinal morphology.

1.4.4 Light intensity

Marine fish larvae that are predominantly visual feeders are dependent upon light intensity for optimal feeding performance. Different species require different light intensities for optimal feeding in laboratory trials (Huse, 1994), such that a prescribed standard for light intensity in larval culture is not appropriate and the requirements of each species need to be assessed. Several authors have acknowledged that the light requirements of cultured larvae may need to be adjusted to correspond with ontogenetic changes in the larval retina (Roo *et al.*, 1999; Shand, 2001). In fact, Pankhurst and Hilder (1998) demonstrated the optimal light intensity for visually mediated feeding behaviour of striped trumpeter larvae changes in conjunction with morphological changes within the retina, such that feeding behaviour ceases above and below a critical light intensity range. This remains to be investigated in a replicated feeding trial.

Objective: Determine the optimal light environment for visual feeding of striped trumpeter larvae in replicated short-term feeding trials.

1.4.5 Greenwater

The addition of microalgae in intensive and extensive finfish culture, known as greenwater culture, has been investigated with several species (cod *Gadus morhua*, turbot *Scophthalmus maximus*, halibut *Hippoglossus hippoglossus* - van der Meeren and Naas, 1997; turbot, Dover sole *Solea solea* - Jones *et al.*, 1981; sea bass *Dicentrarchus labrax* - Cahu *et al.*, 1998; red drum *Sciaenops ocellata* - Lazo *et al.*, 2000). In some studies greenwater has had beneficial effects on

growth and survival of larvae (Naas *et al.*, 1992; Tamaru *et al.*, 1994), while in others it has made no difference compared with clearwater methods (Gulbrandsen *et al.*, 1996). The success of greenwater has been attributed to improved water quality (Alderson and Howell, 1973; Støttrup *et al.*, 1995), reduction in cannibalism (Bristow *et al.*, 1996), improved larval nutrition due to direct consumption of algal cells (Moffatt, 1981; van der Meeren, 1991) or indirectly through live feeds (Jones *et al.*, 1981; Moffatt, 1981; Reitan *et al.*, 1993; Reitan *et al.*, 1997) or increased digestive enzyme activity (Cahu *et al.*, 1998; Lazo *et al.*, 2000), and increased feeding response of larvae (Naas *et al.*, 1992; Lazo *et al.*, 2000). Greenwater also produces a gradient in light intensity and increased turbidity within the culture tanks, which may enhance the ability of larvae and juveniles to detect and capture prey organisms (Boehlert and Morgan, 1985; Naas *et al.*, 1996). A reduction in aberrant larval behaviour (wall-nosing) has been attributed to the changed light distribution in turbid conditions (Bristow and Summerfelt, 1994). Early rearing trials with striped trumpeter indicated greenwater was a promising culture method for this species (Ruwald *et al.*, 1991). This study examined the effect of greenwater on larval feeding performance with particular reference to the light environment in algal cell-induced turbid water.

Objective: Determine the effect of algae-induced turbidity (greenwater) on the feeding performance of striped trumpeter larvae.

1.4.6 Jaw development and malformation

The incidence of jaw malformation in juvenile striped trumpeter cultured to date is almost 100%. Jaw and other skeletal malformations are frequently described in cultured finfish (Barahona-Fernandes, 1982; Andrades *et al.*, 1996). Some skeletal malformations are common between species, for example lordosis and kyphosis due to problems with swimbladder inflation in red sea bream (*Pagrus major*), sea bass and sea bream (*Sparus auratus*) (Kitajima *et al.*, 1981; Chatain, 1994). Development of the jaw appears to be a labile phase in larval development, subject to interference from environmental factors, such that jaw

malformations are common, but manifest in different forms (Hickey *et al.*, 1977; Barahona-Fernandes, 1982; Pittman *et al.*, 1990; Morrison and MacDonald, 1995). Jaw malformations may have lethal or sublethal effects (e.g. poor growth, unsaleable product), through the inability of fish to feed adequately (Barahona-Fernandes, 1982; Pittman *et al.*, 1989). Thus, the malformation in striped trumpeter has the potential to severely impact on the success of culturing this species. The type of jaw malformation observed in striped trumpeter was previously undescribed, and an understanding of the onset of the malformation was required to propose possible causes to the problem. In addition, the effect of this malformation on the feeding success and subsequent viability of young larvae was unknown. Since a large portion of this study involved investigations of larval feeding, it was appropriate to examine the development of the jaw, a vital mechanical structure involved in feeding (Drost, 1987; Lazzaro, 1987; Kohno *et al.*, 1996), and the jaw malformation in cultured striped trumpeter larvae.

Objective: Describe the development of the jaw and characterise jaw malformation in cultured striped trumpeter larvae, with particular reference to possible affects on feeding success.

1.4.7 Animal ethics approval

Experimentation was conducted with the approval of the University of Tasmania Animal Ethics Committee, approval numbers 97049 and A5537.

1.4.8 Thesis structure

The thesis is structured with a general introduction (current chapter), followed by four research chapters, each written in a format appropriate for submission to a scientific journal, and concludes with a general discussion chapter which emphasises the application of the results to the development of rearing techniques for striped trumpeter. Due to the structure of the thesis there may be some replication of introductory information in Chapter 1 and in the

introductions to the research chapters. Chapter 5 has been published in Aquaculture 199(3-4), 267-282. Chapter 4 has been published in Journal of Fish Biology 59(5), 1181-1197.

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2. Chapter Two. Sensory organ development in cultured striped trumpeter larvae *Latris lineata* and implications for feeding behaviour

2.1 Abstract

Teleost larvae are reliant upon sensory organs for feeding, in particular for the detection and subsequent capture of prey. The present study describes the development of sensory organs in cultured striped trumpeter, *Latris lineata*, larvae. In addition, a short-term feeding trial was conducted to examine the feeding response of larvae with different senses available; streptomycin sulphate was used to ablate the superficial neuromasts, while testing larvae in the dark prevented visually mediated feeding. Some non-visual senses are available to striped trumpeter larvae from an early age, indicated by the presence of superficial neuromasts at hatching, and innervated olfactory organs and a developed inner ear from day 3 post-hatching. The neuromasts proliferated on the head and body with increasing larval age, and formation of the lateral line canal had commenced by day 26 post-hatching. Taste buds were not present in any of the larvae examined, up to day 26 post-hatching. At hatching, the retina was poorly developed, but differentiated rapidly and was presumed functional coincident with the onset of feeding on day 7 post-hatching. The ventro-temporal retina was the last to differentiate, and was distorted by the embryonic fissure, such that larval vision in the forward and upward visual field would be compromised. In contrast, the dorso-temporal retina was the first area to differentiate, and presumptive rod and double-cone development occurred in this area from days 11 and 16, respectively, indicating the forward and downward directed visual field is most suited for acute image formation. Larvae on day 18 post-hatching demonstrated increased feeding with an increase in the senses available, with 8 ± 3% of streptomycin-treated larvae feeding in the dark (chemoreception and inner ear mechanoreception only) and 27 ± 5% of untreated larvae feeding in the light (all senses available). It remains to be

demonstrated whether there is an advantage to larval growth and survival by providing live feed during the dark phase in culture, facilitating feeding 24 hours per day.

Keywords: marine fish larvae, vision, neuromast, olfaction, striped trumpeter

2.2 Introduction

The larvae of broadcast spawning marine teleosts generally hatch at a small size and early stage of development that constrains the functional capabilities of the young larvae (Blaxter, 1986; Kotrschal *et al.*, 1990). Larval fish are dependent upon their behavioural capabilities for survival, particularly on their abilities to detect and avoid predators and locate and capture prey. Several studies have demonstrated the rapid morphological development of marine finfish larvae with ontogeny and the associated improvement in their sensory and motor function (Blaxter, 1986; Higgs and Fuiman, 1998). The suite of sensory organs involved in feeding and predator avoidance is species specific (Batty and Hoyt, 1995; Higgs and Fuiman, 1998). Examination of the development of larval sensory structures and how that correlates with changes in behavioural capabilities provides insight into the ecology of larvae in the wild. Such examination also enables the conditions suited to the sensory function of a particular species to be refined for larval culture.

In most instances fish larvae are highly selective planktivores with an absolute reliance upon sensory organs for the detection of prey, prior to eliciting the predatory strike. The sensory structures involved in larval feeding are primarily the eyes, however mechanoreception and chemoreception may also contribute (Blaxter, 1986; Batty and Hoyt, 1995). Demersal and/or nocturnally feeding fishes utilise non-visual senses in the location of prey (Jones and Janssen, 1992; Batty and Hoyt, 1995). Poling and Fuiman (1998) suggested larvae in complex habitats (e.g. seagrass beds) rely on mechanoreception due to their limited visual range, whilst pelagic larvae associated with surface waters of relatively high light intensity use vision.

The striped trumpeter is a candidate species for temperate marine aquaculture. To date, the morphological development of the larvae has been described (Furlani and Ruwald, 1999) and parameters for egg incubation have been examined (Bermudes and Ritar, 1999; Morehead *et al.*, submitted), although larval rearing has proved problematic (Cobcroft *et al.*, 2001b; Trotter *et al.*, 2001). Little is known of the sensory biology of the larvae. The development of the eyes of young larvae has been described (Pankhurst *et al.*, 1998) and the appropriate light intensity for visual feeding determined under culture conditions (Cobcroft *et al.*, 2001a). However, there is no information about the development of non-visual senses or their possible involvement in live prey capture. The aims of this study were to describe sensory organ development in striped trumpeter larvae, and investigate the contribution of sensory organs to larval feeding behaviour.

2.3 Materials and Methods

2.3.1 Larval culture

Striped trumpeter larvae were sourced from four cohorts (cohorts 1 to 4) cultured at the Tasmanian Aquaculture and Fisheries Institute, Marine Research Laboratories (MRL) to examine the development of sensory organs and the relative contribution of sensory organs in feeding behaviour.

Eggs and sperm were hand-stripped from naturally ovulated and spermiated wild-caught broodstock held at the MRL, according to the methods described by Morehead *et al.* (2000). Eggs for cohort 1 (April 1997), were manually fertilised and held in 270-l incubators in recirculating 1 μm filtered seawater, with an upwelling flow of 100-l.h^{-1}. A 24-h light photoperiod was provided by cool white fluorescent globes (Crompton), with a light intensity of 2 μmol.s^{-1}.m^{-2} (100 lux) at the water surface. Larvae hatched 6 days post-fertilisation at 12.9 ± 0.2°C (mean ± SE), and yolksac larvae were stocked into a 1000-l cylindroconical culture tank on day 5 post-hatching at a density of 29 larvae.ml^{-}

[1]. The larvae were reared at 17.5 ± 0.1°C in a static "greenwater" culture in which an algal cell density of 0.33 ± 0.12 x 10^6 cells.ml^{-1} was maintained with a daily water exchange of 150-300-l *Tetraselmis suecica* algae, diluted with 1 μm filtered seawater when necessary. Dissolved oxygen, pH, and total ammonia nitrogen were monitored daily in the greenwater culture and averaged 6.6 ± 0.1 mg.l^{-1}, 7.76 ± 0.04 and, 0.9 ± 0.1 mg.l^{-1}, respectively. Light intensity, provided by an incandescent spotlight (Philips) suspended 1 m above the water surface, was 30 μmol.s^{-1}.m^{-2} (1500 lux) in the centre of the tank at the water surface from days 5 to 15 post-hatching, then reduced to 1 μmol.s^{-1}.m^{-2} (50 lux) from day 16 post-hatching, with a photoperiod of 16-h light/8-h dark throughout the larval rearing period. The bottom of the tank was siphoned daily to remove mortalities and a thorough siphon of the tank bottom was done once a week. Rotifers were added twice daily from day 7 post-hatching, at 10 rotifers.ml^{-1}, and *Artemia* nauplii were added at 1-2 nauplii.ml^{-1} from day 25 post-hatching. An overnight flow rate of 200 l.h^{-1} using 0.1 μm filtered seawater was commenced from day 26 post-hatching to flush out old *Artemia*. The culture was terminated at day 31 post-hatching with 15 surviving larvae. The standard lengths (SL, from rostral tip to end of notochord) of ten live (non fixed) larvae from the culture tank were measured using image analysis on days 2, 4, 6, 8, 12, 16, 19, 21, 23, 26, and 31 post-hatching. Images of whole larvae were captured by a Sony CCD from a Wild M5 stereo microscope, and analysed with Scion ImagePC Beta3b (©Scion Corporation) software.

Cohort 2 (March-April 2000) was reared under similar greenwater conditions to cohort 1.

Cohort 3 (March-April 1998) larvae were sourced from eggs incubated at 12.8°C, before hatching on day 6 post-fertilisation. During incubation, there was a 24-h light photoperiod, with a light intensity of 0.99 μmol.s^{-1}.m^{-2} (20 lux) provided by fluorescent lights (Crompton) covered with a red filter. On day 5 post-fertilisation, eggs were stocked into four 300-l tanks at a density of 47 eggs.l^{-1}. A 12-h light/12-h dark photoperiod from day 5 post-hatching was provided by Osram Biolux® fluorescent lights, with a layer of shade cloth to

moderate light intensity to 1.34 - 2.24 μmol.s^{-1}.m^{-2} (90 - 150 lux) at the water surface. A recirculating system provided a flow of 150 l.h^{-1} of 1 μm filtered seawater at 13.2 ± 0.1°C, and 20% of the total system volume was exchanged daily. Mortalities were siphoned twice a week from the tank bottom. Larvae were fed from day 6 post-hatching on rotifers enriched with DHA Selco® (INVE Aquaculture, Belgium), added twice daily at 10 rotifers.ml^{-1}, and from day 21 post-hatching on newly hatched (Instar 1) AF *Artemia,* at 1-2 nauplii.ml^{-1}. The SL of ten live larvae was measured on each of days 0, 5, 7, 8, 11,15, 20, 21, 26, and 35 post-hatching to determine growth.

Cohort 4 (September-October 1997) larvae were sourced from eggs incubated with 24 hours light, at 2.4-3.6 μmol.s^{-1}.m^{-2} (200-300 lux), before hatching 6 days post-fertilisation at 12.5 ± 0.2°C. Yolksac larvae, 2 days post-hatching, were stocked into a 270-l tank at a density of 200 larvae.l^{-1}, with light aeration and recirculating 1 μm filtered seawater at 12.1 ± 0.0°C providing 55% exchange of tank volume per hour. Maximum light intensity at the water surface was 1.5 μmol.s^{-1}.m^{-2} (65 lux), provided by Biolux® fluorescent globes, and a photoperiod of 12-h light/12-h dark was introduced from day 6 post-hatching. Rotifers boosted with *T. suecica* and/or DHA Selco® were added twice daily from day 7 post-hatching at 10.ml^{-1}.

2.3.2 Anatomy

2.3.2.1 Histology and Scanning Electron Microscopy

Larvae were sampled from cohort 1 on days 3, 5, 6, 8, 14, 16, 19, 21, and 26 post-hatching, and fixed overnight at 4°C in 2.5% glutaraldehyde in 0.1M phosphate buffer containing 2g sucrose per 100ml, pH 7.4. The following day, larvae were rinsed with sucrose-phosphate buffer to remove the fixative, then stored in 70% ethanol at 4°C until processed for histology. The standard lengths of the fixed larvae were measured prior to dehydrating in an ethanol series of 70, 90, 100%, and embedding in glycol methacrylate resin (either JB4 - Agar Scientific, or LKB 2218-500 Historesin). The heads of 19 larvae were serially

sectioned in the transverse plane, using Ralph-Bennett glass knives, to 2 μm thickness. Sections were stained with Lee's Methylene Blue-Basic Fuchsin (Wikeley and Goodsell, 1994) and mounted for light microscopy. An additional 20 fixed larvae were measured, dehydrated in an ethanol series, transferred to acetone, critical point dried (Bal-Tec CPD 030), then mounted and sputter coated with gold, and examined with a Phillips 505 scanning electron microscope (SEM).

Seven larvae from cohort 2, one from each of days 1, 4, 5, 7, 12, 16, and 26 post-hatching, were sectioned in the sagittal plane for light microscopy to examine development in naso-temporal regions of the eye, for comparison with the dorso-ventral regions examined in transverse sections of larvae from cohort 1 (above).

2.3.2.2 Transmission electron microscopy

Larvae were sampled from cohort 3 on days 4, 5, 6, 7, 11, 12, 13, 14, 19, 21, and 26 post-hatching, and fixed for 2-3 hours in 2% glutaraldehyde and 2% formaldehyde in 0.1M phosphate buffer, pH 7.4, containing 2g sucrose per 100ml. Larvae were post-fixed in 1% osmium tetroxide in the sucrose-phosphate buffer for 1-2 hours, rinsed in buffer, then dehydrated in an ethanol series, transferred to acetone and embedded in Procure 812 resin (ProSciTech). This method for optimal fixation of larvae for TEM required immediate transfer of larvae to the fixative and precluded the measurement of individual larvae prior to primary or secondary fixation. Six larvae were sectioned in the transverse plane, and thin sections were stained with uranyl acetate (2-2.5 h) and lead citrate (10-15 min), before examination with a Philips CM 100 Bio TEM.

2.3.2.3 Olfactory organs

Light microscopy and TEM of serial sections of the heads of larvae from cohorts 1 and 2, and SEM of whole larvae from cohort 1 were used to examine and describe development of the olfactory organs. The serial sections observed by light microscopy (cohorts 1 and 2) were also used to determine the presence or absence of taste buds.

2.3.2.4 Mechanoreceptors

The distribution and proliferation of superficial neuromasts were mapped on *camera lucida* drawings of larvae from days 0 to 26 post-hatching, determined by combining observations of live larvae from cohort 4 stained with Janus Green (Appendix 1; Blaxter *et al.*, 1983), serial transverse sections of the heads of larvae from cohort 1, and SEM of whole larvae from cohort 1.

2.3.2.5 Visual morphology and morphometry

Development of the retina of striped trumpeter larvae was described by examination of the eyes of larvae from cohorts 1 and 2 with light microscopy. Dorso-ventral eye and lens diameters were measured at the largest diameter serial transverse sections in the central retina using an eyepiece micrometer fitted to a Nikon Optiphot-2 compound microscope at 400x magnification. Photoreceptor types identified at the light microscope level (cones, double cones, and rods) were confirmed by TEM in the retinae of larvae from cohort 3.

2.3.3 Behaviour

2.3.3.1 Feeding experiment

A single feeding trial was conducted with larvae from cohort 4 on day 18 post-hatching (5.62 ± 0.06 mm SL, live, n = 10) to assess the relative contribution of sensory organs to feeding in larval striped trumpeter. Larvae were treated with streptomycin sulphate to ablate the mechanoreceptive neuromasts (Blaxter and Fuiman, 1989) while leaving the inner ear intact (Matsuura *et al.*, 1971). Treated larvae were then allowed to feed in the light so that vision and chemoreception were available (strep-light), or in the dark so that chemoreception and mechanoreception of the inner ear were available (strep-dark). The feeding behaviour of streptomycin treated larvae was compared with that of untreated, control larvae in the light, with all sensory organs available (light-control), and with untreated larvae in the dark that had mechanoreception and chemoreception available (dark-control). Light intensity was measured with a Li-Cor LI-250

light meter with an LI-192SA sensor and with a Profisix lux meter (Gossen). In the light treatments intensity was 1.3-1.5 $\mu mol.s^{-1}.m^{-2}$ (60-70 lux), within the range determined to be optimal for visual feeding in this species (Cobcroft *et al.*, 2001a). In the dark treatments it was below the sensitivity threshold of the light meters, <0.01 $\mu mol.s^{-1}.m^{-2}$ (<0 lux), and was presumed to be below the visual sensitivity threshold for feeding of striped trumpeter larvae (Pankhurst and Hilder, 1998). The evening before the feeding trial, 30 larvae from the culture tank were added to each of twenty 3-l test chambers, five replicates per treatment. Treated larvae were placed in a bath of 10 $mmol.l^{-1}$ streptomycin sulphate in seawater for 10-15 minutes (Blaxter and Fuiman, 1989), then rinsed in clean seawater before transfer to the test chambers. Untreated larvae were added directly into the test chambers (no sham treatment), and all test chambers were filled to the 3-l level with 1 μm filtered seawater. The chambers were covered with blackout cloth and held in the dark overnight. An additional test chamber containing 30 untreated larvae was included as a control for gut evacuation and was sampled before commencing the feeding trial to ensure that food observed in larvae could be attributed to feeding within the trial. On the day of the feeding trial, rotifers were added to the test chambers at a density of $2.ml^{-1}$, and the larvae were left to feed in the appropriate treatment conditions for 90 minutes. The addition of food was staggered at ten minute intervals to provide time to assess feeding at the end of each replicate's feeding time. At the end of the feeding period for each replicate, larvae were collected on a 100 μm screen, pipetted onto chilled microscope slides, squashed with a coverslip, and examined for evidence of feeding under a dissecting microscope. Feeding was scored for whole rotifers only, because a small number of mastax (rotifer mouthparts) were identified in larvae from the gut evacuation control.

The feeding trial was conducted 16 – 21 h after initial streptomycin treatment, thus some recovery of the neuromasts may have occurred by that time, although recovery appears to be species specific (Blaxter and Fuiman, 1989). Prior tests with striped trumpeter larvae treated with streptomycin showed 50% recovery of responsiveness to a predator stimulus (an advancing probe), in the dark, 19 h

after treatment (Cobcroft, unpubl. data), therefore some effect of neuromast ablation was likely during the present feeding trial.

2.3.4 Statistics

One-way ANOVA was used to examine the effect of treatment on the proportion of larvae feeding and on larval feeding intensity. Cochran's test for homogeneity of variance and the Shapiro-Wilk test for normal distribution of residuals (replicate means - treatment means) were used to determine whether data satisfied the assumptions of ANOVA. Tukey-Kramer multiple comparison of means was used when treatment effect was significant.

2.4 Results

2.4.1 Larval growth

The standard length of larvae reared in higher temperatures (~17°C, cohorts 1 and 2) tended to increase more rapidly with age in degree-days than those reared at 12-13°C (cohorts 3 and 4) (Fig.2.1.). Therefore, both larval age in days and larval length were used in the description of sensory organ development.

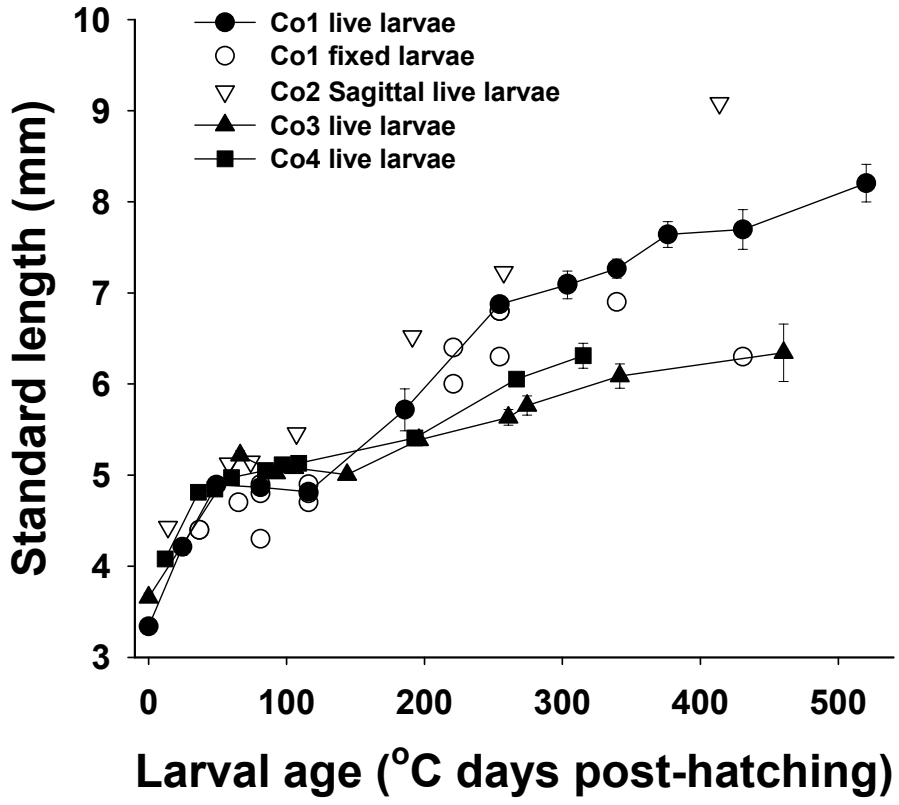

Figure 2.1. Standard length of striped trumpeter larvae in four cohorts reared to examine sensory organ development and the role of sense organs in feeding behaviour. Age is standardised to degree-days to compensate for differences in rearing temperature. Values are means ± SE (n = 10). Cohort 1 (Co1)- open and filled circles, cohort 2 (Co2)- open triangles, cohort 3 (Co3)- filled triangles, cohort 4 (Co4)- squares. Standard lengths of individual larvae prepared for light microscopy are indicated by open symbols; circles and triangles denote larvae sectioned in the transverse and sagittal planes, respectively.

Striped trumpeter larvae hatched at 3.3-3.7 mm SL, live, with a large yolksac and single oil droplet. The yolk was absorbed by day 8 (~4.8 mm SL, live) or day 10 (~5.1 mm SL, live) post-hatching, at 17°C and 12.5°C, respectively. The mouth was open and first feeding was observed on day 7 (~4.8 mm SL, live) at 17°C, and on day 10 (~5.1 mm SL, live) at 12.5°C. The oil droplet was absorbed 1-2 days after first feeding.

2.4.2 Olfactory organs

Two olfactory placodes were present in larval striped trumpeter from hatching, symmetrically arranged on the ventral surface of the head, anterior to the eyes. The olfactory nerve was present from day 3 post-hatching (4.43 mm SL, fixed) (Fig.2.2a). The sensory epithelium of the olfactory organ widened with increasing larval age, and by day 26 post-hatching (6.31 mm SL, fixed) had formed a nasal pit (Fig.2.2b,c,d). The sensory epithelium was comprised of receptor cells, basal cells, supporting cells, and degenerating ('white') cells (Fig.2.2).

Two receptor cell types, microvillous and ciliated, were identified by transmission electron microscopy in the sensory epithelium on day 11 post-hatching (~5.0 mm SL, live) (Fig.2.3a,b). The ciliated receptor cell had a knob projecting from the surface of the olfactory epithelium around which cilia with a 9+2 microtubule arrangement protruded (Fig.2.3b,c). The second receptor cell type had microvilli projecting from the apical surface (Fig.2.3b). Numerous desmosomes, junctions between adjacent (sensory) cell membranes, were apparent in the upper portion (approximately 1.5-2.0 μm) of the olfactory epithelial cells (Fig.2.3d).

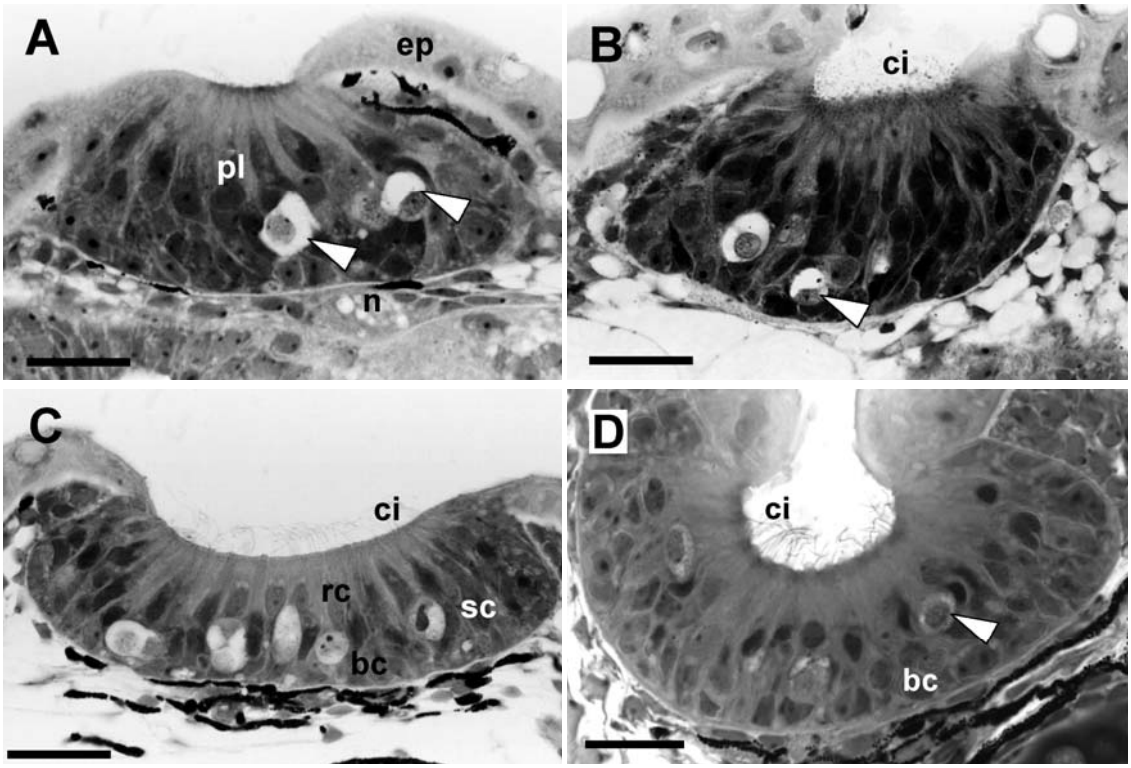

Figure 2.2. Photomicrographs of the development of the olfactory organs of striped trumpeter larvae. Transverse sections of larvae on A) day 3 (4.43 mm SL, fixed), B) day 5 (4.66 mm SL, fixed), C) day 19 (7.15 mm SL, fixed), and D) day 26 post-hatching (6.31 mm SL, fixed). Arrowheads indicate degenerating ('white') cells. Abbreviations: bc, basal cell; ci, cilia; ep, epidermal epithelium; n, olfactory nerve bundle; pl, placode; rc, receptor cell; sc, supporting cell. Scale bars are 20 μm.

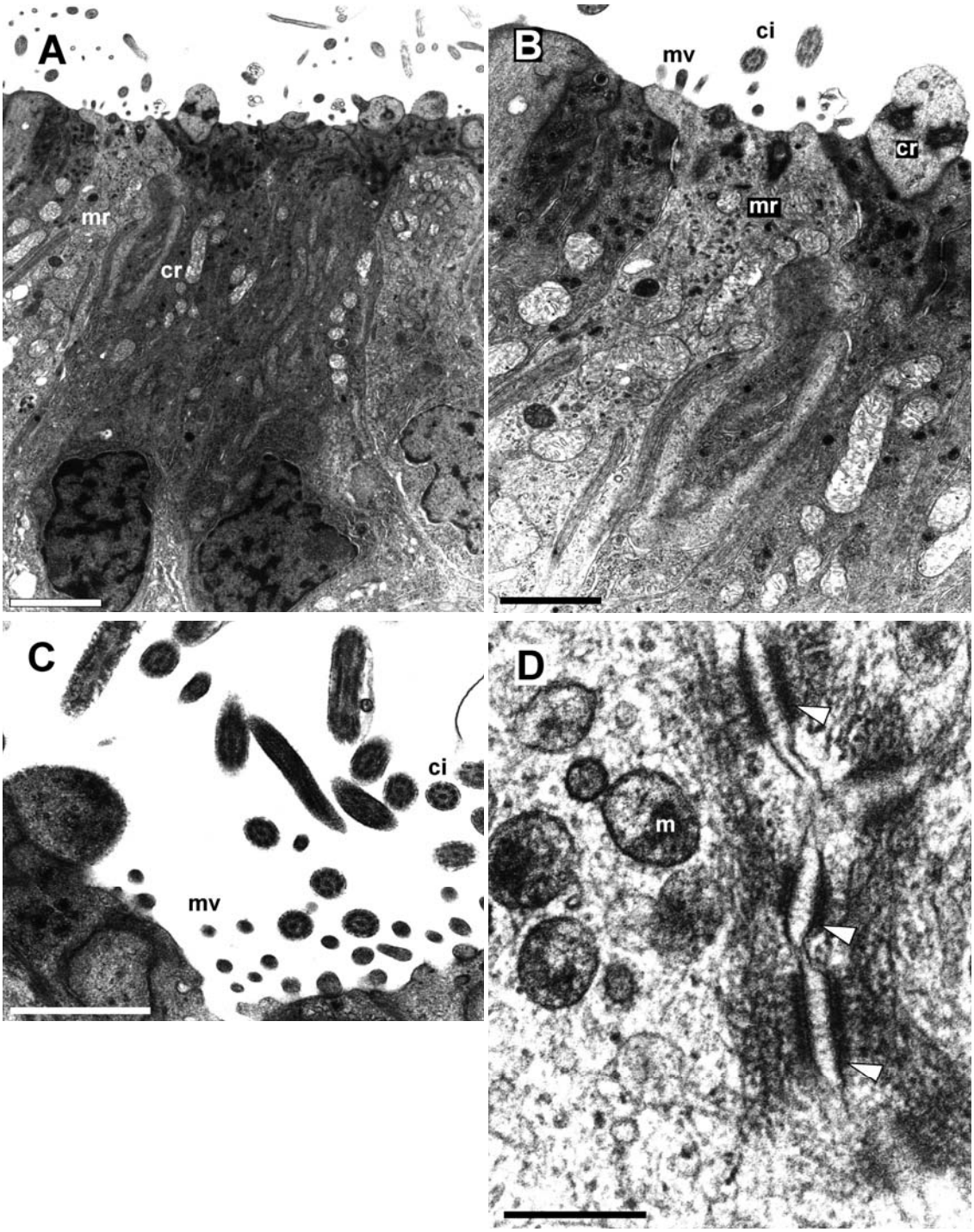

Figure 2.3. Transmission electron micrographs of the sensory epithelium of the olfactory organs of striped trumpeter larvae. Day 11 larva (~5.0 mm SL, live) A)&B) apical surface of the olfactory epithelium showing microvillous (mr) and ciliated (cr) sensory cell types, C) 9+2 microtubule arrangement in cilia, and D) desmosome junctions (arrowheads) between neighbouring cells. Abbreviations: ci, cilia; m, mitochondria; mv, microvilli. Scale bars indicate A) 2 μm, B)&C) 1 μm, and D) 200 nm.

2.4.3 Mechanoreceptors

At hatching, striped trumpeter larvae had two pairs of superficial neuromasts on the head and one pair on the body (Fig.2.4a). On the head, one large pair was located dorso-temporal to the eyes, and the second smaller pair was found at the anterior of the head dorso-medial to the olfactory placodes. On the body, the neuromasts were located medially on the trunk and level with the centre of the yolksac. The neuromasts proliferated with increasing larval age; on the head, forming a line along the upper and lower jaws, and a ring around the eyes, and forming a line along the length of the body (Fig.2.4). Superficial neuromasts were arranged asymmetrically along the left and right hand sides of the body (Fig.2.4e). The neuromasts consisted of support cells and receptor hair cells, with long kinocilia and short stereocilia projecting from the apical surface (Fig.2.5a,b). In live larvae, stained with Janus green, long club-shaped cupulae were observed projecting from the superficial neuromasts, covering the cilia. These cupulae were removed during fixation and processing of fixed specimens. On day 26 post-hatching (7.18 mm SL, fixed), an opening of the lateral line canal was apparent at the posterior end of the trunk with a superficial neuromast located above the opening (Fig.2.5c). No other superficial neuromasts were observed at this stage, however lateral line canal formation was evident on the head (Fig.2.4h).

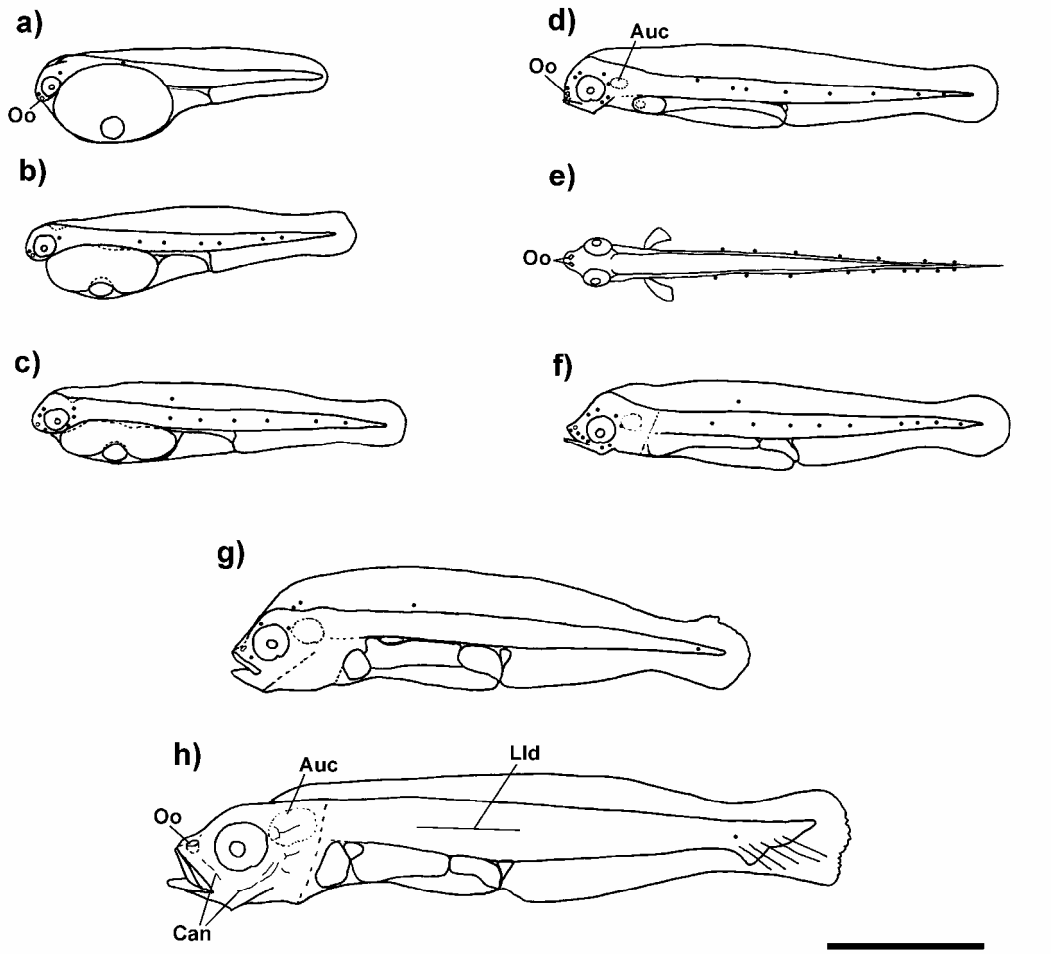

Figure 2.4. *Camera lucida* drawings of striped trumpeter larvae indicating the distribution and proliferation of superficial neuromasts (black dots) with age. a) day 0, 3.5 mm SL, b) day 1, 4.0 mm SL, c) day 2, 4.6 mm SL, d) day 8, 5.3 mm SL, e) day 13, 5.4 mm SL, f) dorsal view showing asymmetric neuromast locations on the left and right hand sides of the body on day 13, 5.4 mm SL, g) day 14, 6.4 mm SL, and h) day 26, 8.3 mm SL. Standard lengths (SL) are of fixed larvae. Abbreviations: Auc, auditory capsule; Can, enclosed lateral line canals; Lld, lateral line depression; Oo, olfactory organ. All figures are the same scale, and scale bar is 2 mm.

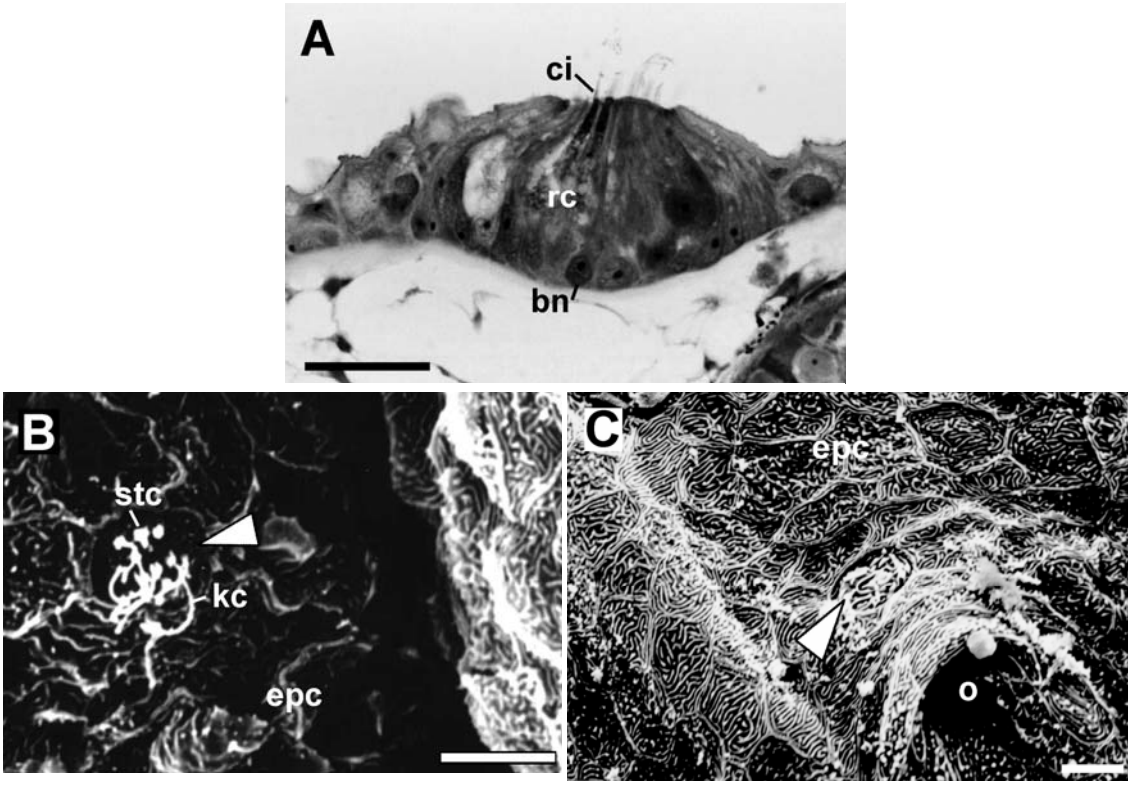

Figure 2.5. Superficial neuromasts of striped trumpeter larvae. A) Photomicrograph of a transverse section of a neuromast located dorso-temporal to the eye of a day 5 larva (5.15 mm SL, live), and scanning electron micrographs of B) neuromast on the head of a day 8 larva (4.82 mm SL, fixed) with kinocilia and stereocilia projecting from the apical surface of the hair cells, and C) a superficial neuromast above the posterior end of the lateral line canal opening of a 26 day old larva (7.18 mm SL, fixed). Arrowheads indicate neuromasts. Abbreviations: bn, basal nuclei of support cell; ci, cilia bundle; epc; epidermal epithelial cells; kc, kinocilium; o, lateral line canal opening; rc, receptor cell; stc, stereocilia bundle. Scale bars A) 20 μm and B)&C) 10 μm.

On day 3 post-hatching (4.43 mm SL, live), the sensory tissue on the ventral surface of the utriculus chamber of the inner ear consisted of support and sensory hair cells (Fig.2.6). Cilia projected from the apical surface of the sensory cells and were in contact with the lapillus (otolith) at this stage.

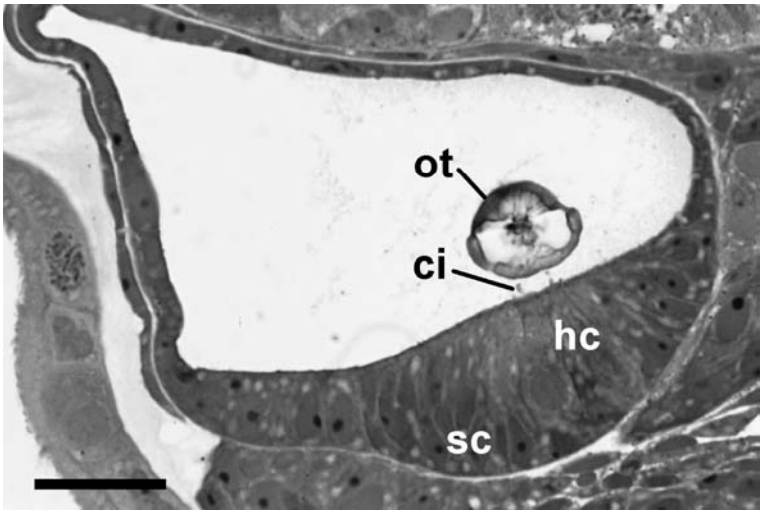

Figure 2.6. Photomicrograph of a transverse section of the head of a 3 day old striped trumpeter larva (4.43 mm SL, fixed), through the inner ear with cilia from hair cells in contact with the lapillus otolith within the utriculus chamber of the labyrinth. Abbreviations: ci, cilia; hc, sensory hair cells; ot, otolith; sc, support cell. Scale bar is 20 μm.

2.4.4 Visual morphology

At hatching (day 0, 2.71 mm SL, fixed), the eyes of striped trumpeter larvae were poorly developed as the lens consisted of a concentric arrangement of non-crystalline fibroblasts, and radially arranged neuroblasts were apparent in the presumptive non-pigmented retina (Fig.2.7a). Differentiation of cell layers within the retina was evident from day 1 post-hatching (4.43 mm SL, live), particularly in the dorso-temporal region of the retina. The optic nerve (ON) was located in the ventro-medial region of the developing retina, near the widest eye diameter, and connected with the optic tectum in a 3 day old larva (4.43 mm SL, fixed) (Fig.2.7b). The outer nuclear layer (ONL), inner nuclear layer (INL), and the ganglion cell layer (GCL) were identified in the differentiating retina on day 3, demarcated by the inner and outer plexiform layers (IPL and ONL, respectively) (Fig.2.7b). Further differentiation of cells in the ONL, INL, and GCL was evident from day 4 post-hatching (~4.9 mm SL, live) (Fig.2.7c&d), and transmission electron microscopy revealed horizontal cells were developing in the INL, along with presumptive cone photoreceptor pedicles (synapses), nuclei, and ellipsoids in the ONL (Fig.2.8a). Pigmentation of the retina was initiated from day 4 post-hatching in the dorso-temporal region of the retina (Fig.2.7c) and was complete by day 8 post-hatching, when the ventro-nasal region became pigmented (Fig.2.9a). Also in 8 day old larvae, a pure single cone photoreceptor layer was present, with outer segments (OS) projecting to the pigment epithelium (PE), the GCL, INL, and ONL were differentiated, and a germinal cell layer persisted at the retina margin (*ora terminalis*) (Fig.2.9a&b). At this stage, the lens had a crystalline core surrounded by a layer of fibre cells. The retractor lentis muscle (RLM) was present from day 4 post-hatching. In 8 day old larvae there was a vitreous space around the lens in the centre of the eye of some larvae (Fig.2.9a&c). However, the lens remained in close contact with the retina at the nasal and temporal poles in the histological sections, connective tissue appearing to adhere the lens to the retina in some sections (Fig.2.9d). A pigmented intrusion into the ventral retina was observed from day 8, the embryonic fissure (Fig.2.9c), through which a blood vessel supplying the RLM and the ON passed.

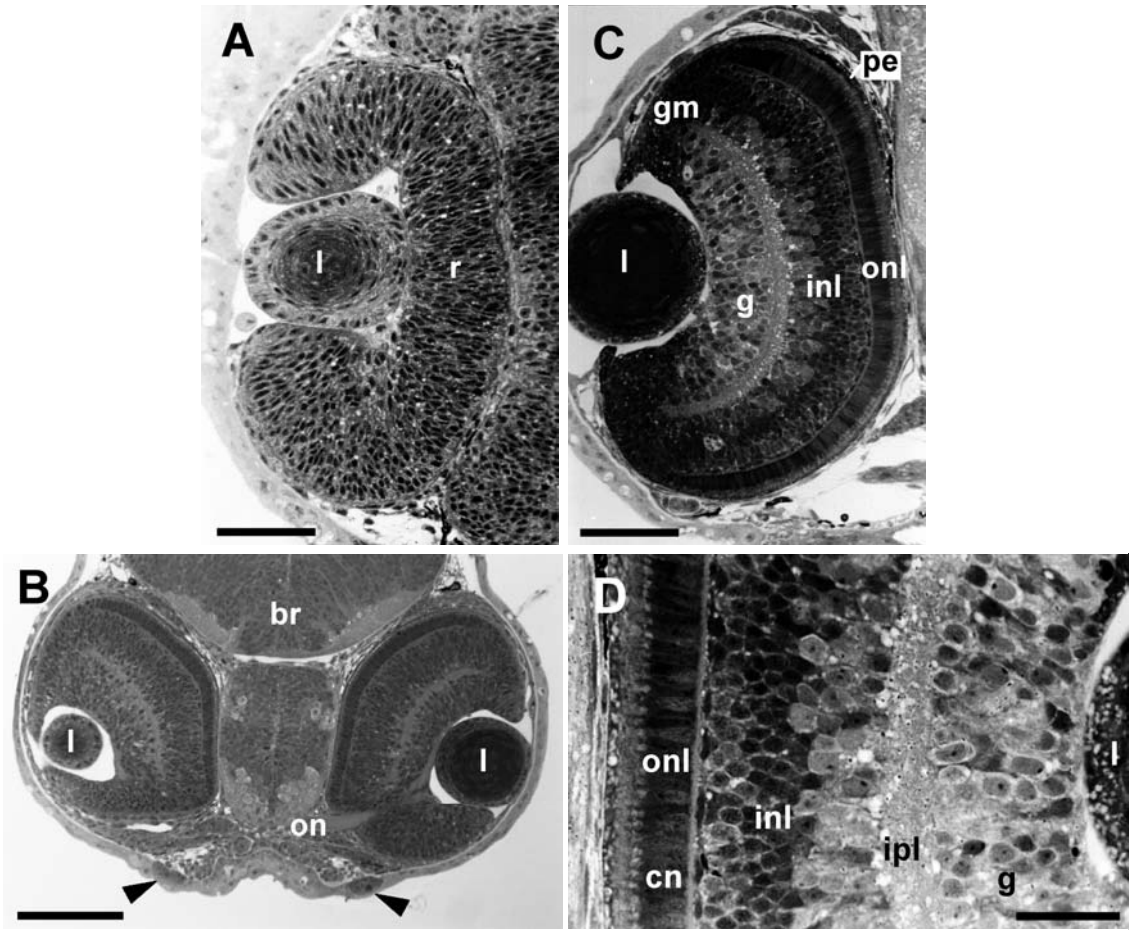

Figure 2.7. Photomicrographs of transverse sections through the developing eyes of striped trumpeter larvae. A) Differentiating neuroblasts in the retina and fibroblasts in the lens of a larva at hatching (day 0, 2.71 mm SL, fixed). B) Position of the optic nerve in a 3 day old larva (4.43 mm SL, fixed). C) & D) Development of layers in the retina of a 5 day old larva (4.66 mm SL, fixed). All figures are orientated with dorsal upwards. Arrowheads indicate neuromasts. Abbreviations: br, brain; cn, cone nuclei; g, ganglion cell layer; gm, germinal cell margin; inl, inner nuclear layer; ipl, inner plexiform layer; l, lens; on, optic nerve; onl, outer nuclear layer; pe, pigmented retinal epithelium; r, retina. Scale bars in A)&C) are 50 μm, in B) 100 μm, and in D) 20 μm.

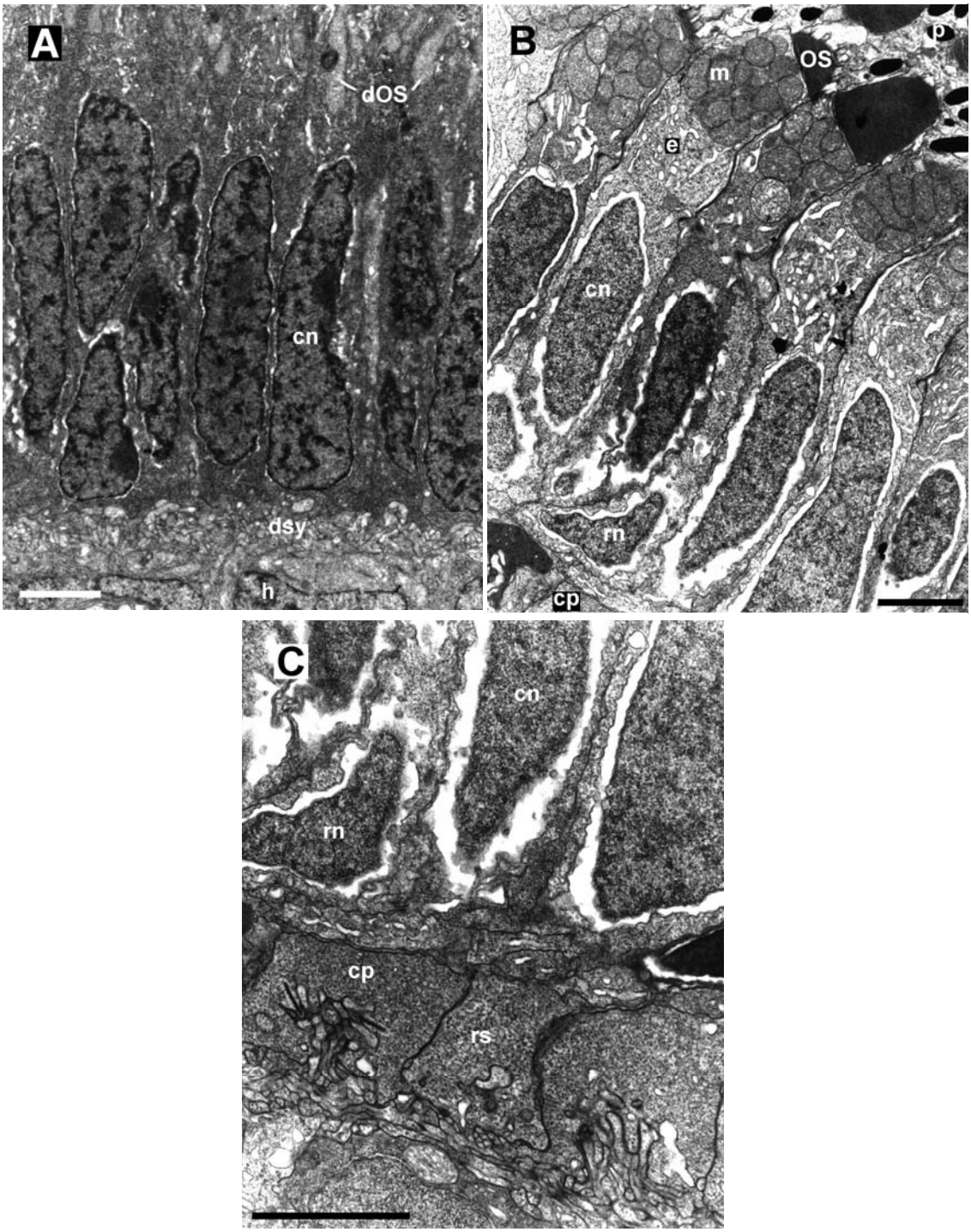

Figure 2.8. Transmission electron micrographs of A) developing photoreceptors in a 4 day old larva (~4.9 mm SL, live), and in an 11 day old larva (~5.0 mm SL, live) B) differentiated single cone photoreceptors, and C) cone synapses. Abbreviations: cn, cone nuclei; cp, cone pedicle; dOS, differentiating outer segments; dsy, developing synapses; e, cone ellipsoid; h, horizontal cell; m, mitochondria; OS, outer segments; p, pigment of the pigmented retinal epithelium; rs, rod spherule. Scale bars are 2 μm.

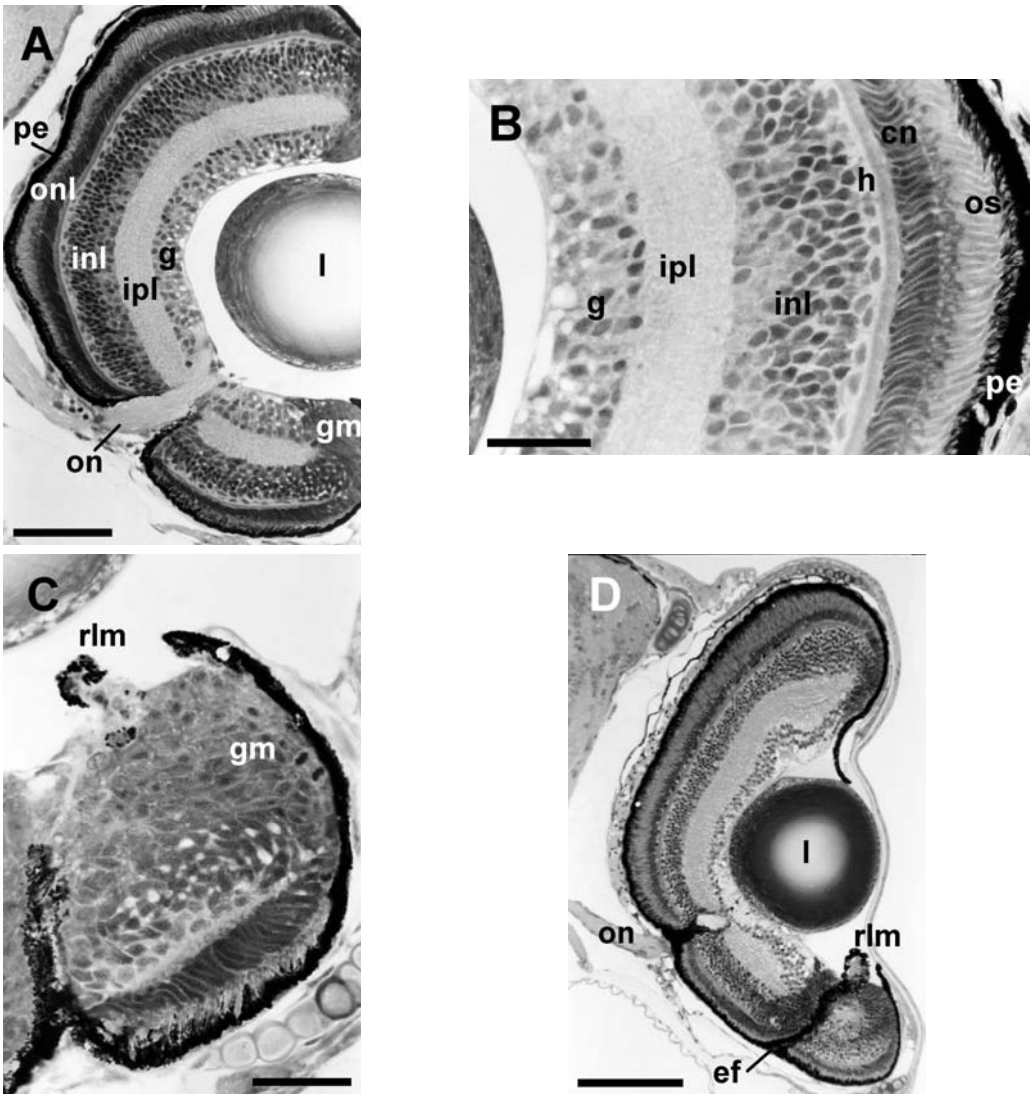

Figure 2.9. Photomicrographs of transverse sections through the retina of striped trumpeter larvae on day 8 post-hatching (4.94 mm SL, fixed), demonstrating A) the position of the optic nerve and a crystalline lens, B) differentiated retinal layers and single cone photoreceptors, C) the retractor lentis muscle and the embryonic fissure ventral to the lens, and D) on day 21 post-hatching (6.95 mm SL, fixed) showing the extent of distortion in the ventral retina due to the embryonic fissure. All figures are orientated with dorsal upwards. Abbreviations: cn, cone nuclei; ef, embryonic fissure; g, ganglion cell layer, gm, germinal cell margin; h, horizontal cell; inl, inner nuclear layer; ipl, inner plexiform layer; l, lens; onl, outer nuclear layer; os, outer segment layer; pe, pigment epithelium; rlm, retractor lentis muscle. Scale bar in A) 50 μm, B)&C) 20 μm, and D) 100 μm.

The embryonic fissure was associated with distortion of the retinal layers throughout most of the ventro-temporal retina in older larvae (Fig.2.9d). The neural cells around the embryonic fissure were not differentiated, similar in appearance to the cells of the germinal margin. The adjacent photoreceptors and pigmented epithelium were bent into the retina, following the path of the embryonic fissure, such that the outer segments were orientated at up to 90 degrees to their normal position. From day 14 post-hatching, the cone ellipsoids and outer segments were longer in the dorsal retina than in the ventral retina, exemplified by the distance from the external limiting membrane to the pigment epithelium on day 14 measuring approximately 25 μm and 16 μm, respectively (Fig.2.10a&b). Rod precursor nuclei and double cones were identified in light micrographs of the retinae of larvae from days 14 (6.38 mm SL, fixed) and 16 (6.75 mm SL, fixed) post-hatching, respectively, being more prevalent in the dorsal than in the ventral retina (Fig.2.10). However, transmission electron micrographs indicated presumptive rod development, by the presence of rod spherules and nuclei (Fig.2.8b,c), as early as day 11 post-hatching (~5.0 mm SL, live). On day 21 post-hatching, the photoreceptors were more dense (Chapter 3, section 3.4.2) and the outer segments were staggered in the dorsal retina, while an aligned single row of outer segments, closely associated with the pigment epithelium, was present in the ventral retina (Fig.2.10c&d).

In the temporal retina, a bulge in the photoreceptor and pigment layers was visible in sagittal sections of larvae from day 4 post-hatching (5.12 mm SL, live), the *area temporalis* (Fig.2.11a&b). In older larvae (days 16 and 26 post-hatching, 7.23-9.08 mm SL, live) a high density of long photoreceptor OS were present in the area.

Tangential sections through the photoreceptor layer revealed a possible linear pattern to the arrangement of single cones in 8 day old larvae (Fig.2.12a). A square mosaic of central single cones surrounded by presumptive double cones, more clearly defined in the dorsal region compared with the ventral, was present in 26 day old larvae (Fig.2.12b).

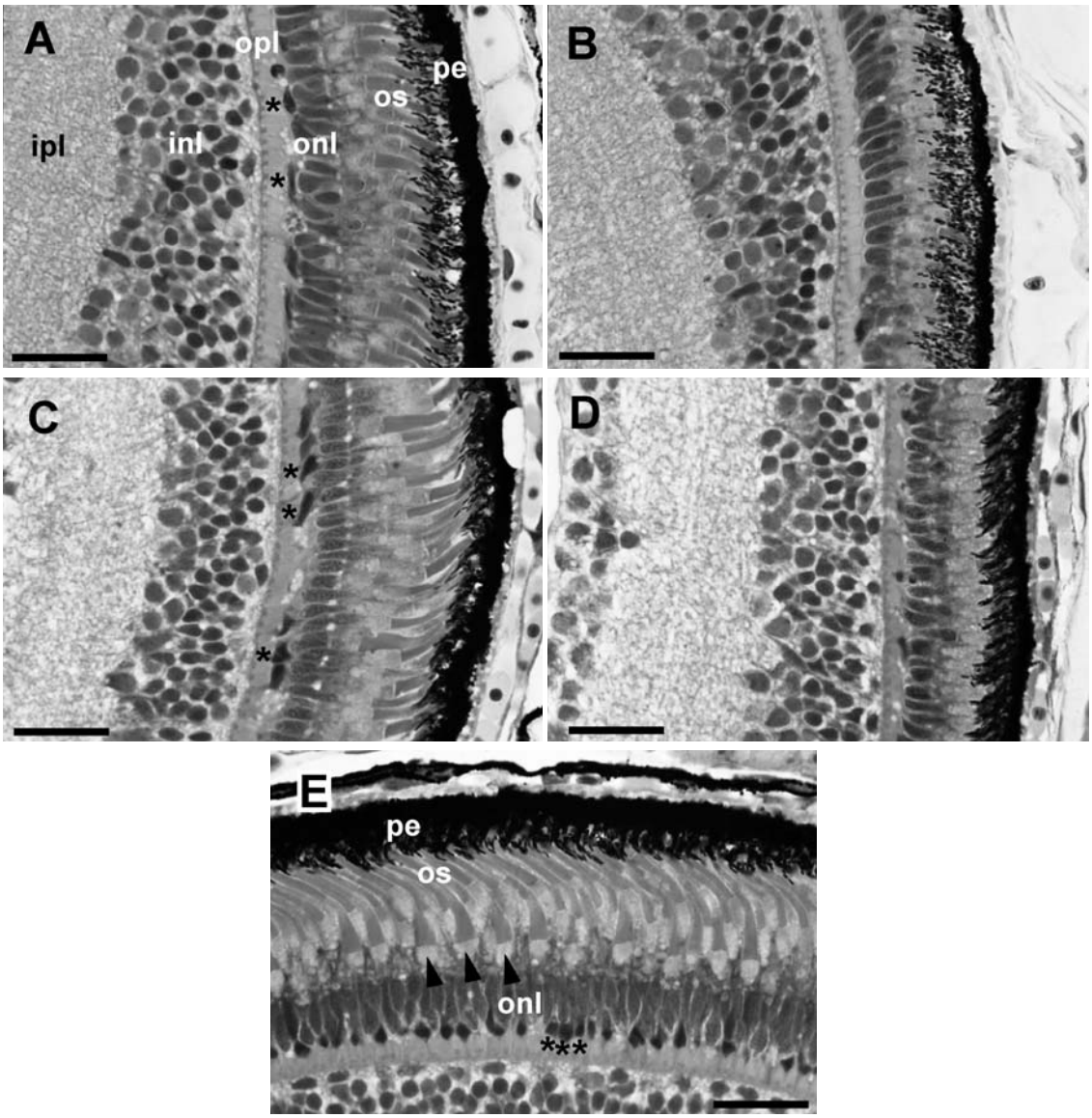

Figure 2.10. Photomicrographs of transverse sections through regions of the retina of striped trumpeter larvae on day 14 post-hatching (6.38 mm SL, fixed) A) dorsal and B) ventral, on day 21 post-hatching (6.95 mm SL, fixed) C) dorsal and D) ventral, and E) on day 26 post-hatching (9.08 mm SL, live) dorsal. Figures A) to D) are orientated with sclerad to the right, and E) is sclerad upwards. Presumptive rod nuclei are indicated by *, and presumptive double cones by arrowheads. Abbreviations: inl, inner nuclear layer; ipl, inner plexiform layer; onl, outer nuclear layer; opl, outer plexiform layer; os, outer segment layer; pe, pigment epithelium. Scale bars are 20 μm.

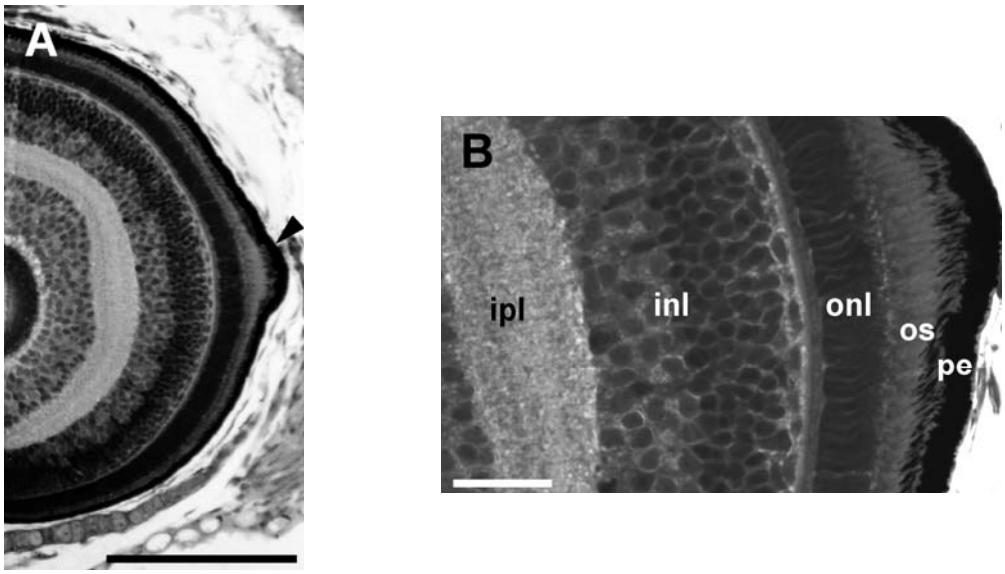

Figure 2.11. Photomicrographs of sagittal sections through the eye of a 5 day old larva (5.15 mm SL, live), showing A) advanced pigmentation and retinal layer development in the dorso-temporal retina and an *area temporalis* (arrowhead) associated with a bulge in the outer segment (os) and pigment epithelial (pe) layers, and B) a magnified view of the *area temporalis*. Abbreviations: inl, inner nuclear layer; ipl, inner plexiform layer; onl, outer nuclear layer. Scale bars A) 100 μm and B) 20 μm.

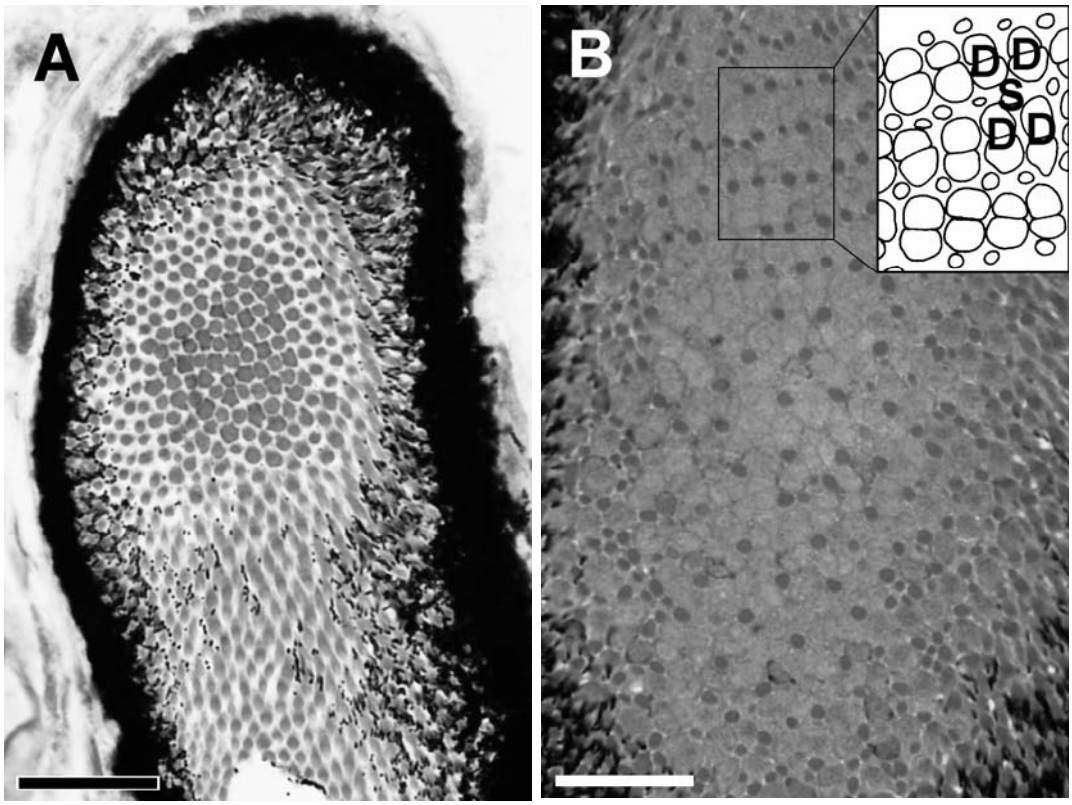

Figure 2.12. Photomicrographs of transverse sections through photoreceptor ellipsoids and outer segments in the retinae of striped trumpeter larvae on A) day 8 post-hatching (4.94 mm SL, fixed) revealing an almost linear arrangement of single cones, and B) day 26 post-hatching (6.31 mm SL, fixed) revealing a square mosaic in the dorso-temporal retina. The position of double cones (D) relative to the central single cone (S) is marked in the inset in B). Scale bars are 20 μm.

Eye and lens diameters, measured in the dorso-ventral plane in transverse histological sections, increased in a linear fashion with larval age (Fig.2.13). Between day 3 (4.40 mm SL, fixed) and day 21 (6.95 mm SL, fixed) post-hatching, eye and lens diameter had more than doubled from 0.23 to 0.56 mm and from 0.08 to 0.18 mm, respectively.

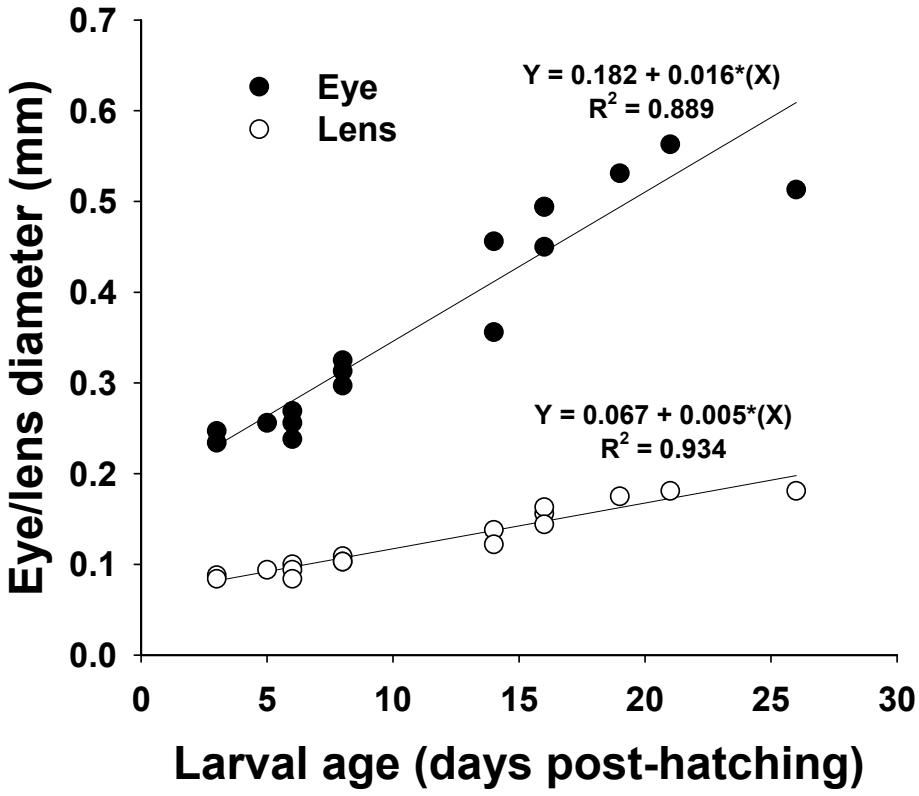

Figure 2.13. Eye and lens diameters of striped trumpeter larvae with increasing age. Measurements were made in the dorso-ventral plane in transverse histological sections. Values are from individual fish.

2.4.5 Behaviour

The proportion of striped trumpeter larvae feeding on day 18 post-hatching was significantly affected by the suite of sensory organs available for feeding (ANOVA, F = 3.67, df 3, 16, P = 0.035). The proportion of larvae feeding was lowest in the strep-dark treatment (with chemoreception and inner ear mechanoreception only available to the larvae), higher in the dark-control treatment (with neuromast mechanoreception functional), higher again in the strep-light treatment (with vision only), and highest in the light-control treatment (with all sensory organs functional) (Table 2.1). However, the proportion of larvae feeding in the light-control was only significantly higher than that in the strep-dark treatment (P<0.05). There were no significant differences between feeding intensity in any of the treatments tested, with between 1 and 2.4 rotifers consumed per feeding larva in the 90 minute feeding time.

Table 2.1. Feeding response of 18 day old striped trumpeter larvae after 90 min exposure to rotifer prey in the light and in the dark, with and without treatment with streptomycin sulphate to ablate neuromasts. Larval standard length, 5.62 ± 0.06 mm, live. (n = 5 replicates, with 30 larvae per replicate.)

	Proportion larvae feeding (%)			**Feeding intensity (rotifers consumed per feeding larva)**	
Treatment (sense organs available)	mean	±SE		mean	±SE
Streptomycin sulphate – dark (Chemoreceptors, inner ear)	8	3	a	1.0	0.4
Dark – control (Chemoreceptors, inner ear, lateral line)	15	4	ab	1.9	0.6
Streptomycin sulphate – light (Vision, chemoreceptors, inner ear)	19	4	ab	2.4	0.3
Light – control (All)	27	5	b	2.2	0.3

a, ab, b - Different letters indicate differences between means, significant at P = 0.05.

2.5 Discussion

2.5.1 Olfactory organs

Striped trumpeter larvae appear to have functional olfactory organs from an early age, day 3 post-hatching, prior to first-feeding, indicated by the presence of the olfactory nerve and a ciliated sensory epithelium. In rainbow trout (*Oncorhynchus mykiss*), greater development of the olfactory organs occurs prior to hatching, as the olfactory placodes sink into the epithelium, and formation of nares is coincident with hatching (Hara and Zielinski, 1989). In this study, an olfactory pit had formed by day 26 post-hatching, the oldest larvae examined, but the timing and mechanism of enclosure of the olfactory placode remain to be determined. The northern anchovy (*Engraulis mordax* - O'Connell, 1981), Dover sole (*Solea solea* - Appelbaum *et al.*, 1983), Atlantic halibut (*Hippoglossus hippoglossus* - Døving and Knutsen, 1993), and greenback flounder (*Rhombosolea tapirina* - Pankhurst and Butler, 1996), have a ciliated olfactory epithelium, presumed functional prior to first-feeding, similar to that of striped trumpeter. This is consistent with the known role of chemoreception in larval activity and distribution prior to feeding (Dempsey, 1978; Tanaka *et al.*, 1991). Tanaka *et al.* (1991), suggested there may be an energetic advantage to yolksac larvae locating and maintaining position in a prey patch in the wild, as this would enhance prey encounter rates and searching efficiency once feeding commenced. Prey searching activity and ingestion may also be mediated by chemical stimuli in young, feeding teleost larvae (Dempsey, 1978; Knutsen, 1992; Batty and Hoyt, 1995; Kolkovski *et al.*, 1997). Dempsey (1978) demonstrated behavioural responses of larvae are elicited by chemicals isolated from prey species that the larvae are experienced with and not by chemicals from prey species to which larvae are naive. The latter indicates sensory learning affects larval responses and is likely to improve the incidence of prey encounters.

The ultrastructure of the sensory epithelium of striped trumpeter olfactory organs is similar to that found in teleosts generally, consisting of microvillous

and ciliated sensory neurons (Zeiske *et al.*, 1992). Thommesen (1983) provided evidence that microvillous cells are specific to amino acids, while ciliated cells are specific to bile salts, and Hara and Zielinski (1989) found the abundance of these cell types varied in different regions of the developing olfactory organ in rainbow trout, with ciliated cells found on the anterior edge and both cell types in the middle. The 9+2 microtubule arrangement in the olfactory cilia of striped trumpeter is similar to most teleosts (Yamamoto, 1982), although some have a 9+0 arrangement (Zeiske *et al.*, 1976). Likewise, desmosomes (junctions) observed in striped trumpeter, are common between cells of the sensory epithelium associated with tonofilament bundles within the cells and provide structural support. Asymmetric junctions have been observed between sensory and supporting cells, while symmetric junctions, similar to those identified in this study, occur between receptor cells (Zeiske *et al.*, 1976).

Taste buds were not identified in the striped trumpeter larvae examined, up to day 26 post-hatching, which is consistent with the findings of Iwai (1980). Iwai (1980) observed that the differentiation of taste buds appears slower in marine species than in freshwater species, with no taste buds present until well after the onset of first-feeding in some pelagic marine larvae. This indicates the gustatory sense does not play a major role in feeding during the early larval stages of some marine larvae, including striped trumpeter. In contrast, older fish use taste buds to determine palatability and either accept or reject items taken into the mouth (e.g. juvenile plaice *Pleuronectes platessa* - Batty and Hoyt, 1995).

2.5.2 Superficial neuromasts

Superficial (‘free’) neuromasts were present in striped trumpeter larvae at hatching, suggesting the larvae were capable of movement detection at that time. Teleost larvae use mechanoreception to respond to predator stimuli (detect predators), and in older fish they are involved in prey detection and orientation of the striking action in some species (Janssen and Corcoran, 1993). The proliferation of superficial neuromasts in striped trumpeter was similar to that found in other marine teleost species, however numbers along the trunk did not

reach one per myotome as in herring (*Clupea harengus* - Blaxter and Fuiman, 1989), nor doublet and triplet formation as seen in Atlantic croaker (*Micropogonias undulatus* - Poling and Fuiman, 1997). Poling and Fuiman (1998) associated the abundance of superficial neuromasts in three sciaenid species with their habitats, suggesting that the species living in seagrass (spotted seatrout, *Cynoscion nebulosus*), a structurally complex environment, had an advantage in predator and prey detection due to higher numbers of superficial neuromasts on the head. In striped trumpeter larvae undergoing flexion, 26 days post-hatching, the presence of an opening (pore) at the posterior end of the body, a reduction in the number of superficial neuromasts on the trunk, and a lateral depression along the body, indicates the trunk canal was forming at that stage. Lateral line canals on the head were also present in 26 day old larvae. Incorporation of neuromasts into canals is thought to reduce their sensitivity to the increasing amount of 'noise' generated by the swimming motion of developing larvae and thus increase sensitivity to movement from other sources (Bleckmann, 1993). In striped trumpeter, trunk canal formation was coincident with flexion, a stage when the swimming pattern of larvae changes (Batty, 1984), and is likely to maintain the mechanoreceptive ability of the larvae.

The inner ear of striped trumpeter was likely functional for the maintenance of equilibrium by day 3 post-hatching, suggested by the presence of cilia on the surface of the hair cells of the utriculus sensory epithelia in contact with the otolith (lapillus) at that time (Bond, 1996). Kawamura and Ishida (1985) found cilia were not present in the inner ear of larval Japanese flounder (*Paralichthys olivaceus*) at hatching, but the organ was ciliated 6 hours post-hatching, coinciding with horizontal orientation of larvae. Striped trumpeter larvae are largely inactive on day 3 post-hatching, floating passively with their heads down, however on day 4 they become more active and maintain a horizontal orientation (Trotter, pers. comm.). This suggests the functional ability to react to sensory input from the inner ear occurs later than the development of the sensory structure.

2.5.3 Visual morphology

Development of the eyes in striped trumpeter larvae was generally similar to that observed in other pelagic teleost larvae that hatch with unpigmented poorly developed eyes, including herring and Dover sole (Sandy and Blaxter, 1980), northern anchovy (O'Connell, 1981), Japanese flounder (Kawamura and Ishida, 1985), New Zealand snapper (*Pagrus auratus* - Pankhurst and Eagar, 1996), greenback flounder (Pankhurst and Butler, 1996), red porgy (*Pagrus pagrus* - Roo *et al.*, 1999), and black bream (*Acanthopagrus butcheri* - Shand *et al.*, 1999a). The eyes of striped trumpeter larvae were presumed functional by day 8 post-hatching since there were three differentiated retinal neural layers present (GCL, INL, and ONL), two plexiform layers (IPL and OPL), the optic nerve was in contact with the optic tectum, the lens was crystalline, and the retina was pigmented. The retractor lentis muscle was also present at this stage, enabling movement of the lens in the vitreous space of fish eyes to achieve fine focus (Fernald, 1989). Differential shrinkage of the retina and lens may have occurred during preparation of the histological material, such that the lens appeared closer to the retina than would actually be the case (Shand *et al.* 1999b). However, movement of the lens was likely constrained in the larvae examined, since all had lenses at some point contiguous with the vitread surface of the neural retina, such that the lens did not sit free within the vitreous space. While the contact of the lens with the retina may have precluded dynamic accommodation (the ability to maintain focus on objects moving within the visual field), Shand *et al.* (1999b) suggested variability in the focal ratio in young larvae enables the temporal retina to focus close while the remainder may focus distant objects prior to the ability to accommodate. The development of the eyes and the onset of first-feeding in larvae in this study were comparable to that observed in the same species by Pankhurst and Hilder (1998). The latter reported that the retina was functional at day 6 post-hatching, one day prior to first feeding in larvae incubated and reared at 14°C, similar to larvae from cohort 2 sampled for sagittal sectioning, while the retinas of larvae reared at a lower temperature were not functional until day 7 or 8 post-hatching. Presumptive rod nuclei and double cone photoreceptors were present at an earlier age and at a smaller size in this

study (day 11 post-hatching, ~5 mm SL, live, and day 16 post-hatching, 6.8 mm SL, fixed, respectively), compared with the results of Pankhurst and Hilder (1998) (day 23 post-hatching, 7.5 mm TL, fixed, and day 25 post-hatching, 7.1 mm TL, fixed, respectively, lengths from Butler, 1995). These contrasting results may be due either to the different rearing regimes, particularly incubation in the dark in Pankhurst and Hilder (1998) compared with in the light in the present study, and different culture temperatures, or to the ages of larvae examined in each study.

The development of a square mosaic of single and double cones from a linear arrangement of single cones in the retina of striped trumpeter larvae is consistent with observations in the black bream (Shand *et al.*, 1999a). Shand *et al.* (1999a) demonstrated that the formation of subsurface cisternae along neighbouring cell membranes was associated with the reorganisation of a linear mosaic to an adult-type square mosaic. Cone mosaics in fishes may be responsible for increased acuity, colour discrimination, movement detection, and sensitivity (Kunz *et al.*, 1983; Fernald, 1989). Several visual pigments have been identified in the different types of photoreceptors of larval marine fish, including yellow-, green-, blue-, violet-, and ultraviolet-sensitive cones, even when only a single morphological type of cone was present (Britt *et al*., 2001). Thus, it is highly likely that the different cone types identified in striped trumpeter larvae contain different visual pigments. Since the cone mosaic of striped trumpeter larvae was more clearly defined in the dorsal compared with the ventral region it is likely these larvae are more sensitive to image forming light projecting onto the dorsal retina.

The pattern of retinal pigmentation, differentiation and specialisation suggests that the dorso-temporal retina is primarily equipped for acute visual function in striped trumpeter larvae, and would translate to a functional visual field in a forward directed, lateral and ventral visual field, which is also the relative position of the mouth. In addition, the position and extent of the embryonic fissure in the ventral retina of striped trumpeter larvae would likely compromise image formation from light originating from the dorso-nasal visual field. The

distortion of the retinal layers and the orientation of the photoreceptors adjacent to the fissure would alter the angle that incident light reaches the photosensitive outer segments, preventing normal image formation. Kunz and Callaghan (1989) suggested the persistence of the embryonic fissure in adult brown trout (*Salmo trutta*), with well-defined photoreceptor layers, may be involved in the detection of polarised light. In contrast, the fissure in other species closes during development, or persists without photoreceptor layers (Kunz and Callaghan, 1989). The structure of the fissure in older striped trumpeter remains to be investigated.

The presence of the *area temporalis* in striped trumpeter larvae, similar to that observed in northern anchovy larvae by O'Connell (1981), indicates particularly acute image formation could occur in the forward and lateral visual field. Similar specialised areas for acute image formation are known in adult teleosts (Tamura, 1957; Tamura and Wisby, 1963) and have been associated with the functional visual field (visual axis) of feeding fish (Raveendran and Mohideen, 1986). Likewise, the functional visual field of feeding striped trumpeter larvae, being forward directed in the horizontal plane (Chapter 3), is consistent with histological observations of the retina in this study.

Increases in eye and lens diameter, such as those seen in this study, are common in developing fish larvae (Pankhurst and Butler, 1996; Pankhurst and Eagar, 1996). The small eye size of many pelagic marine larvae imposes constraints upon how the eye develops in order to achieve maximal acuity at an early stage (Pankhurst *et al.*, 1993; Pankhurst, 1994), either for predator avoidance or for prey detection (Kotrschal *et al.*, 1990). Single cone photoreceptors are packed into the small retina to optimise acuity, however the optics of eye size dictate acute image formation is much better in a larger eye where more photoreceptors receive incident light (Fernald, 1985). Consequently, the ontogenetic increase in eye size improves acuity (Pankhurst *et al.*, 1993; Pankhurst, 1994) and enhances visually mediated behaviours, including feeding (Breck and Gitter, 1983).

2.5.4 Contribution of sensory organs to feeding behaviour

A low proportion of larvae feeding in this study and a slow rate of digestion, as evidenced by incomplete gut evacuation by larvae overnight prior to commencement of the feeding trial, indicates that the results should be interpreted with caution. Notwithstanding this, the present study demonstrated larval striped trumpeter will feed using all senses available to them. Feeding of larvae in the dark with chemoreceptors and mechanoreceptors available indicated non-visual senses may be responsible for prey detection and low levels of feeding in the dark. The suite of sensory organs involved in feeding behaviour of other larvae varies with age and between species. In North Sea turbot (*Scophthalmus maximus*) and Dover sole yolksac larvae, chemoreception was demonstrated to mediate behaviour typically associated with feeding, indicating this sense plays a role in the feeding of these larvae from an early stage (Knutsen, 1992). In contrast, Salgado and Hoyt (1996) observed that first-feeding in larval fathead minnows (*Pimephales promelas*) was reliant upon vision and mechanoreception, and feeding mediated by olfaction was delayed. Kolkovski *et al.* (1997) studied sea bream larvae (*Sparus auratus*) from day 20 to day 35 post-hatching and found vision and chemoreception were active in stimulating their feeding.

A lower proportion of striped trumpeter larvae fed in this trial (27% in light-control larvae) than in subsequent feeding trials looking at the effects of the light environment on larval feeding (53 to 97% in treatments with similar light intensity and feeding duration - Cobcroft *et al.*, 2001a, and Chapter 4). Also, the difference in feeding performance of untreated larvae in the light compared with the dark was much lower in this study than in other feeding trials with larvae of the same species (55-100% in light and 0-2% in dark - Pankhurst and Hilder, 1998 and; 60-76% in light and 15% in dark, Cobcroft, unpubl. data). This disparity in feeding performance may reflect that the current cohort of larvae were less robust than those used in previous (Pankhurst and Hilder, 1998) or subsequent feeding trials (Cobcroft *et al.*, 2001a). Although Pankhurst and Hilder (1998) and the present study indicate vision is the primary sensory modality involved in the feeding behaviour of striped trumpeter larvae, further

trials with more robust larvae, controls for the handling of streptomycin treated larvae, and incorporating video analysis (see Chapter 3) will be required to determine the relative contribution of different senses. Given that striped trumpeter larvae did feed in the dark, the provision of live feed during the dark phase in culture may provide an advantage to the larvae, in terms of growth and survival.

2.5.5 Summary

The early development of sensory organs in striped trumpeter was similar to other pelagic species. Neuromasts were present from hatching likely functioning to detect predators and initiate an avoidance response. The olfactory organs were presumed functional before feeding and may play a role in the location of prey patches. Rapid differentiation of the retina from hatching enabled visually mediated feeding to occur just prior to the endogenous yolk reserve being completely absorbed. Formation of an enclosed lateral line coincided with caudal fin flexion and likely a concomitant change in swimming mode. Results of the feeding trial demonstrated that striped trumpeter may have the capacity to detect prey using all senses available, however it is likely that pre-flexion larvae predominantly feed visually during daylight.

2.6 Acknowledgments

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3. Chapter Three. Characterisation of the visual field of cultured striped trumpeter larvae using analysis of feeding behaviour and histological measures

3.1 Abstract

Theoretical visual acuity of striped trumpeter *Latris lineata* larvae, determined from lens size and cone cell density, improved from 1°17' minimum separable angle (MSA) on day 8 to 39' MSA on day 26 post-hatching. Photoreceptor cell density and morphology of the retinal layers, suggested that the dorsal, dorso-temporal, and temporal regions of the retina appeared specialised for visual sensitivity and acute image formation, corresponding to a forward and downward directed visual field. This was confirmed by the functional visual field in the horizontal plane, determined from analysis of larval feeding behaviour, which was forward and laterally directed. The functional visual field was relatively small compared to larval body size. The maximum reactive distances (the distance at which the predator first reacts to the prey) were 5.07mm and 5.25mm on days 13 and 17 post-hatching, respectively, 97% of larval standard length. The larvae displayed a saltatory searching pattern, periodically stopping to scan for prey throughout the visual field, and exhibited a side-to-side movement of the head as they approached a detected prey item. Thus, pre-strike distance (the distance from the larva to the prey immediately before the strike) may represent the point at which an acute image of the prey is formed and where its location is precisely determined by the larva. Pre-strike distance was 0.44mm and 0.46mm on days 13 and 17, respectively, 8% of larval standard length. The functional visual field expanded laterally with ontogeny as a wider range of reactive angles was used by 17 day-old than 13 day-old larvae, with higher reactive distances within those angles. This increase would substantially increase the area larvae search for prey, and as such suggests prey density requirements may be lower in older larvae. Functional visual acuity, determined from different prey dimensions, was less than the theoretical values,

ranging from 1°41' to 3°25' with reactive distance to 19° to 38° with pre-strike distance.

Keywords: Striped trumpeter, marine fish larvae, visual field, feeding behaviour, retina, visual acuity, prey capture

3.2 Introduction

Most marine fish larvae are highly selective visual planktivores, reliant upon the structural composition of the eye, retina, and higher order neural pathways to interpret visual signals from their environment (Blaxter, 1986; Fernald, 1989). However, marine fish larvae commonly hatch at a small size and early stage in development, which constrains their functional capabilities during feeding (Blaxter, 1986). Kotrschal *et al.* (1990) suggest small eye size limits the concomitant development of daytime (photopic) and night time (scotopic) visual capabilities in very small larvae. As a result, most first-feeding marine fish larvae have a single type of photoreceptor in the eye (the single cone) that provides for visual prey detection under photopic conditions (Blaxter, 1986). As fish grow, visual acuity and sensitivity under high and low light conditions are enhanced by increasing eye size, cone cell enlargement, formation of double cones and the recruitment of rod photoreceptors to the retina (Fernald, 1985; Powers and Raymond, 1990). Within the constraints of small eye and lens size, first-feeding fish larvae optimise daytime visual capabilities by the very close packing of small single cone photoreceptors in the retina (Kotrschal *et al.*, 1990; Pankhurst *et al.*, 1993; Pankhurst and Butler, 1996; Pankhurst and Eagar, 1996). However, resolution is likely to be poor because any image projected by a small lens onto the retina of a small eye is sampled by fewer photoreceptors than in a larger eye and image quality is therefore compromised (Fernald, 1989). Kotrschal *et al.* (1990) suggest resolution is only just adequate for visual planktivory in such small predatory larval fish.

Theoretical estimates of photopic visual acuity, known as minimum separable angle (MSA), that take into account cone spacing in the retina and focal length

of the lens (Neave, 1984) confirm the predictions of Kotrschal *et al.* (1990). In first-feeding fishes, MSA is around 1-2° (Neave, 1984; Pankhurst *et al.*, 1993; Pankhurst, 1994; Job and Bellwood, 1996), which is relatively poor in comparison with the acuity of adults which can reach between 3' and 5' visual angle in carnivorous species (Pankhurst, 1989; Pankhurst and Eagar, 1996). The improvement in MSA with ontogeny tends to be rapid, as increasing cone spacing is compensated for by increasing lens size in the calculation of acuity, and approaches an asymptote (Blaxter and Jones, 1967; Pankhurst, 1994; Pankhurst and Eagar, 1996). Some adult fishes have morphological differences between retinal regions, such that particular regions are more specialised for acute image formation than others (Tamura and Wisby, 1963). For example, pelagic piscivores tend to have specialisation in the temporal retina corresponding to a forward-directed visual field (Pankhurst, 1989).

The visual capability of fish can be modelled using retinal morphology. For example, acuity calculated from retinal morphology can be used to predict the distance at which a fish will respond (known as reactive distance) to a prey item of a given size (Browman *et al.*, 1990; Pankhurst, 1994). Some studies have used behavioural methods such as the optokinetic response to measure the functional visual acuity of fish larvae. In the optokinetic method, the smallest width of black and white stripes rotated around a larval chamber that elicits a response from larvae, such as changed swimming speed and direction, corresponds to the smallest angle subtended at the eye which can be resolved (Neave, 1984; Pankhurst, 1994). Fewer studies have assessed larval visual abilities in terms of the feeding response of the larval fish predator to a prey, specifically reactive distance (Wanzenböck and Schiemer, 1989; Job and Bellwood, 1996; Wanzenböck *et al.*, 1996). Behavioural methods confirm that the functional acuity of larval fishes is low, indeed much poorer than theoretical methods predict (Neave, 1984; Pankhurst *et al.*, 1993; Pankhurst, 1994; Job and Bellwood, 1996), and the reactive distances of larvae to prey are small, in the order of a body length (Blaxter, 1986). However, functional acuity improves with ontogeny more rapidly than theoretical MSA and approaches or exceeds the theoretical value in older fish (Neave, 1984; Pankhurst *et al.*, 1993; Pankhurst,

1994; Wanzenböck *et al.*, 1996). Functional reactive distances throughout the visual field of older fishes have been measured from observations of feeding behaviour and compared with the theoretical capabilities of different regions of the retina (e.g. Browman *et al.*, 1990).

Larvae of the striped trumpeter *Latris lineata*, a candidate species for temperate marine aquaculture, were used to compare acuity determined from behavioural and theoretical methods. Striped trumpeter are broadcast, pelagic spawners, with buoyant eggs, approximately 1.2mm diameter (Morehead *et al.*, 2000). Larvae hatch at 3.2-3.6mm SL with non-pigmented eyes and a large yolksac (Ruwald *et al.*, 1991; Pankhurst and Hilder, 1998; Bermudes and Ritar, 1999; Furlani and Ruwald, 1999). The larvae are primarily visual feeders, and the differentiated structure of the retina, with a pure single cone photoreceptor layer, is suited to functional image formation around the time of first-feeding (Pankhurst and Hilder, 1998; Chapter 2). This study compares the theoretical acuity of striped trumpeter larvae determined using histological measures of lens size and retinal cone density (Neave, 1984) with direct behavioural measures (functional acuity) using videocinematography of feeding larvae to determine the distance between the prey and predator prior to the predator strike. The functional visual field in the horizontal plane was determined by mapping the combined directional responses of larvae of a given age to their prey and was compared with the visual field predicted from retinal morphology.

3.3 Materials and Methods

3.3.1 Larval culture

Striped trumpeter larvae from two cohorts cultured at the Tasmanian Aquaculture and Fisheries Institute, Marine Research Laboratories (MRL) were used for the determination of the theoretical visual field of larvae via histological assessment of the retina (cohorts 1 and 2). A third cohort was reared

for the description of the functional visual field of feeding larvae via analysis of video footage (cohort 3).

Cohort 1 (March-April 1997) larvae were reared according to the method described in Chapter 2 for histological examination of transverse sections of the retina. Briefly, eggs from wild-caught acclimated broodstock were manually collected, fertilised and incubated for 6 days at 12.9 ± 0.2°C before hatching. Larvae were stocked into a 1000-l culture tank on day 5 post-hatching and reared in a static 'greenwater' (containing microalgae) culture at 17.5 ± 0.1°C, 16-h light/8-h dark photoperiod, with daily water exchanges of 15-30% via addition of *Tetraselmis suecica* algae. Light intensity at the water surface was 30 $\mu mol.s^{-1}.m^{-2}$ (1500 lux) until day 15, then reduced to 1 $\mu mol.s^{-1}.m^{-2}$ (50 lux) from day 16 post-hatching. Larvae were fed rotifers (*Brachionus plicatilis*) from day 7 post-hatching, and *Artemia* from day 25 post-hatching. Ten larvae from the culture tank were sampled on days 2, 4, 6, 8, 12, 16, 19, 21, 23, 26, and 31 post-hatching to measure their standard length (SL, from rostral tip to end of notochord). Images of whole larvae were captured by a Sony CCD camera from a Wild M5 stereo microscope, and SL was determined using Scion ImagePC Beta3b (©Scion Corporation) image analysis software.

Cohort 2 (March-April 2000) was reared under similar greenwater conditions to cohort 1 for assessment of the retina sectioned in the sagittal plane.

Cohort 3 larvae (November 1998) were sourced from gametes manually stripped from two female and three male wild-caught, acclimated broodstock. The fertilised eggs were incubated at a temperature of 12.7 ± 0.1°C in a recirculating system providing a flow of 100 $l.h^{-1}$ with a light intensity of 2 $\mu mol.s^{-1}.m^{-2}$ (100 lux) and a 12-h light/12-h dark photoperiod. On the day prior to hatching, day 5 post-fertilisation, eggs were disinfected in a bath of 400ppm glutaraldehyde for 10 minutes and rinsed in 0.2 μm filtered seawater, before being stocked at 47 $eggs.l^{-1}$ into a 300-l cylindroconical tank. The larviculture tank had black sides and a white base, and was illuminated by an overhead Osram Biolux® fluorescent light, covered with shade cloth to reduce the light intensity to 2.3

μmol.s^{-1}.m^{-2} (100 lux) at the water surface while maintaining a broad spectral composition from 400 to 700nm (Appendix 2). Larvae were reared under a 12-h light/12-h dark photoperiod and salinity of 34.8 ± 0.2mg.l^{-1} (mean ± SE). A flow rate of 150 l.h^{-1} of 1 μm filtered seawater, at 13.9 ± 0.1°C, was provided by a recirculating system with a biofilter and protein skimmer. Mortalities were siphoned from the tank bottom twice a week before the addition of live food and daily thereafter. Oily films on the water surface were removed by a surface skimmer from day 8 post-hatching. Larvae were fed twice daily with DHA Selco® (INVE Aquaculture, Belgium) boosted rotifers at a density of 5 rotifers.ml^{-1}. The SL and dorso-ventral eye diameter were measured using image analysis in ten larvae sampled from the culture tank at hatching (day 0), first-feeding (day 8), and on filming occasions (days 13 and 17 post-hatching).

3.3.2 Retinal morphometry

Larvae from cohort 1 were sampled on days 8 (n=3), 14 (n=2), 16 (n=3), 19 (n=1), and 21 (n=1) post-hatching, fixed, embedded in resin (either JB4 - Agar Scientific, or LKB 2218-500 Historesin), and their whole heads were sectioned in the transverse plane at 2 μm for light microscopy, according to the methods described in Chapter 2. Retinal cell counts were made in one eye of each larva in the section with the largest eye diameter (the medial section), and in nasal and temporal sections, approximately 22 μm on either side of the medial section. Only transverse sections where the full length of the photoreceptor nuclei were visible (ie. not oblique sections) were counted. In each of these sections, the number of cone ellipsoids (double cone ellipsoids counted as one unit), photoreceptor nuclei (outer nuclear layer, ONL), horizontal, bipolar & amacrine cells (inner nuclear layer, INL), and ganglion cells (ganglion cell layer, GCL) were counted in the dorsal, central (fundal), and ventral portions of the eye in 50 μm linear transects of retina using 1000x magnification and an eyepiece micrometer fitted to a Nikon Optiphot-2 compound microscope. For consistency, cell fragments were counted at the right-hand-side of transects but not on the left-hand-side. The counts were not corrected for counting bias on the basis of

cell size, because relative differences in retinal region and ontogenetic changes in cell density were the focus of this study, rather than absolute determination of cell numbers (see Higgs and Fuiman, 1996). This method potentially provided cell counts in nine retinal regions (dorso-nasal, nasal, ventro-nasal, dorsal, fundal, ventral, dorso-temporal, temporal, and ventro-temporal; Fig.3.1). One transect in each retinal area was counted in younger larvae (days 8 and 14 post-hatching), while two transects for each of the 9 areas, were counted in older larvae (days 16 to 21 post-hatching) to determine the density of photoreceptor cells and higher order neural processing cells in different areas of the retina.

The number of transects varied with larval age because eye size increased with age and provided a larger distance within which to conduct replicate transect counts. Areas distorted by the optic nerve and embryonic fissure were not

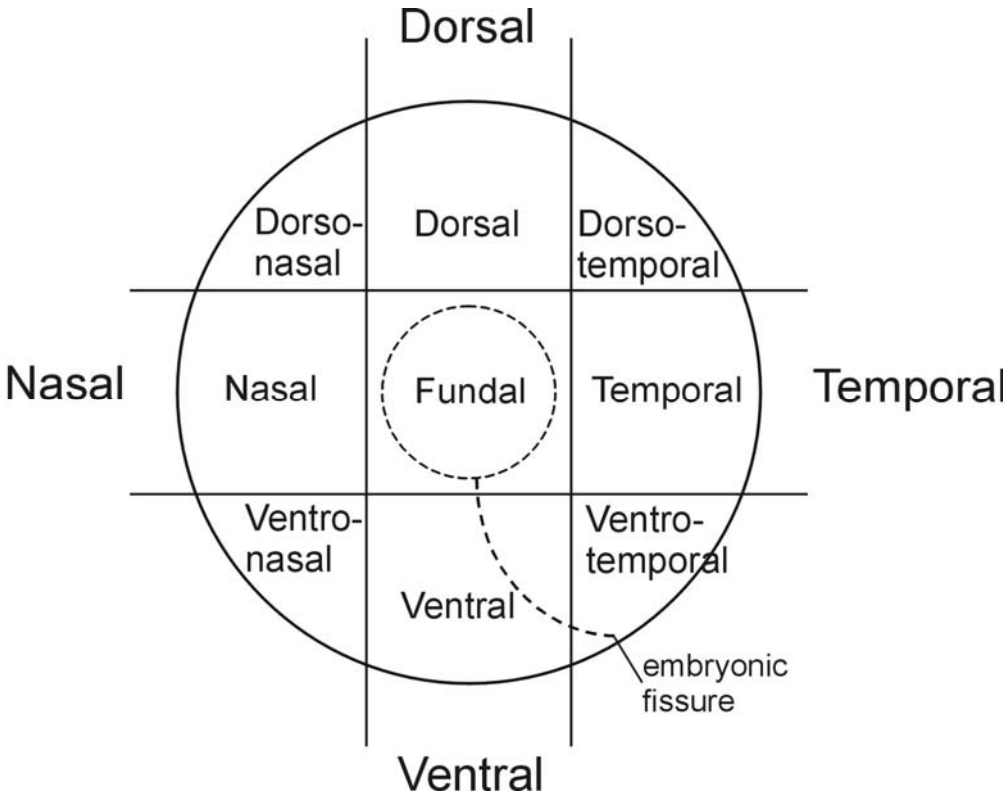

Figure 3.1. A diagrammatic lateral view of the eye of a striped trumpeter larva (large circle) indicating the division of the retina into nine regions (dorso-nasal, nasal, ventro-nasal, dorsal, fundal, ventral, dorso-temporal, temporal, and ventro-temporal) examined for photoreceptor and neural cell density and retinal layer thickness. The small dashed circle indicates the position of the lens and the dashed line denotes the position of embryonic fissure insertion.

counted, causing values to be missing in some retinal areas of some larvae. In order to confirm patterns of regional specialisation in the retina observed in transverse sections, one larva from each of days 7, 12, 16, and 26 post-hatching from cohort 2 was fixed, embedded in JB4 resin and whole bodies were sectioned at 2 μm in the sagittal plane. Cell counts were made as described above for the transverse sections, in the largest diameter section of one eye for each larva. Where the retina was not distorted by the optic nerve and embryonic fissure, one transect was counted in each of eight regions of the eye (dorso-nasal, nasal, ventro-nasal, dorsal, ventral, dorso-temporal, temporal, and ventro-temporal; Fig.3.1) on days 7, 12 and 16, and two transects were counted per region on day 26. The fundal region was not sampled in the sagittal sections, because the lens filled the central field in the largest diameter sections. Also, sagittal sections through the fundal region would be tangential to each of the retinal layers, due to retinal curvature, requiring a different method to count the cells.

The number of presumptive rod nuclei was calculated by subtracting the number of cone ellipsoids from the total number of photoreceptor nuclei. Linear densities of rods, cones, and ganglion cells were converted to angular cell densities using the formula of Poling and Fuiman (1998):

$$\phi = 2.\arctan \{h \div (f - v)\} \qquad (1)$$

where ϕ is the angle subtended by the retinal transect, h is half the transect length, f is the lens focal length estimated by multiplying the lens radius r by 2.55 (Matthiessen's ratio, cited in Neave (1984), and v is the distance between the ganglion cell layer and the external limiting membrane (v is only included when calculating ganglion cell angular density, and adjusts for distortion of ganglion cell densities in very small eyes – see van der Meer, 1993; Poling and Fuiman, 1998). The convergence of photoreceptors onto ganglion cells was determined by calculating the ratio of angular densities of photoreceptor nuclei:GCL, presumptive rod nuclei:GCL, and cones:GCL.

The theoretical acuity of larvae was calculated using linear cone density and the focal length of the lens from the formula of Neave (1984) for minimum separable angle (MSA), incorporating a shrinkage factor of 10% during histological preparation:

$$\alpha = \arcsin \{1.11 \div (10d \times f)\} \qquad (2)$$

where α is MSA, and d is the number of cone cells per 100 μm of retina.

The relative thickness of the retinal layers GCL, inner plexiform layer (IPL), INL, ONL + outer plexiform layer (OPL), and photoreceptor ellipsoids plus outer segments (OS) plus pigment epithelium (PE), was determined by measuring the layer thicknesses (Helvik and Karlsen, 1996), using an eyepiece micrometer and 400x magnification. In the transverse nasal, medial and temporal sections, measurements were made on nine lines projected at 90° to the retina curvature and approximately 30 μm apart, three lines each in the dorsal, medial, and ventral retina, avoiding the germinal cell layer at the retina margins. In the sagittal sections, measurements were made on 11 or 12 lines, at 90° to the retina curvature, one line in each of the nasal, dorsal, temporal, and if possible ventral areas, and 2 lines for the other retinal regions. OS + PE is the distance available for light capture by visual pigments, hereafter referred to as the "light path length" (Pankhurst *et al.*, 1993).

3.3.3 Video cinematography

A black and white, high resolution Ikegami ICD-42B CCD surveillance camera with a 50mm Pentax lens was positioned 21cm above the water surface of the cohort 3 culture tank. Larval behaviour was recorded on a Panasonic HS950 s-VHS video recorder on days 13 and 17 post-hatching for three hours on each occasion, commencing approximately 2h after the onset of the light period, and 1h after live rotifer feed addition at a density of $5.ml^{-1}$. The video recordings were reviewed and larval feeding sequences were selected where the length of the larva was in focus throughout the feeding action. Using this method, the

functional depth of field of the camera was 2.9mm. Striped trumpeter larvae tended to feed near the surface, and the population of larvae in the culture tank on filming days was approximately 3000-6000, such that overall density was 10-20 larvae.l^{-1}. It was assumed that each feeding event observed was from a different larva due to the movement of the larvae by water flow. The 'in tank' recording of feeding events was used because striped trumpeter larvae did not readily adapt and initiate feeding in a small-volume filming arena, and this method enabled the characterisation of the visual field in a culture situation. The outcome from each feeding sequence was categorised as either: caught, when the rotifer was consumed; missed, when the rotifer was still visible after the strike action; turned away, when the larva formed an S-pose in front of the rotifer but did not strike instead turning away from the prey; or uncertain, when the pre-strike position of predator and prey were captured on video but the strike occurred out of the camera's field. Single frames from the feeding sequences recorded on video were captured using AIGotcha95 v.2.03 (©AITech International Co.) video capture card and software. The distance between the rotifer and the rostral tip of the larva, known as the reactive distance (RD), and the angle between the forward directed axis of the larva and the rotifer position, known as the reactive angle (RA), were measured in the frame immediately before the larva turned to pursue the rotifer (Fig.3.2a) (for definitions see Confer *et al.*, 1978; Wanzenböck and Schiemer, 1989). The distance between the rostral tip of the larva and the rotifer position, termed the pre-strike distance (PS) (Job and Bellwood, 1996), was determined in the frame immediately before the larva struck at the rotifer (Fig.3.2b). Measurements were made using Scion ImagePC, calibrated to a 2mm grid that was filmed at the start of the video recording. In the absence of simultaneous dorsal and lateral camera recordings, it was not possible to determine the orientation of larvae and rotifer prey items relative to the horizontal. Nevertheless, where orientation was not horizontal RD and PS would be conservative measurements with a maximum error of 15% underestimation for the largest RDs measured (due to the 2.9mm depth of field), while in the horizontal plane distances measured would be accurate.

Sequences with uncertain outcomes yielded RD, RA and/or PS details, but were not included in the calculation of the proportion of feeding sequences with particular outcomes.

Functional visual acuity determined from feeding behaviour (VA) was calculated with the following equation from Breck and Gitter (1983) using prey size and maximum RD for each age of larvae examined:

$$VA = 2 \arctan (0.5H \div RD) \qquad (3)$$

where H is the prey size. Maximum RD was used because it is thought to provide a better estimate of the capabilities of larvae using a saltatory search pattern, where the larvae stop and search the entire visual field such that the

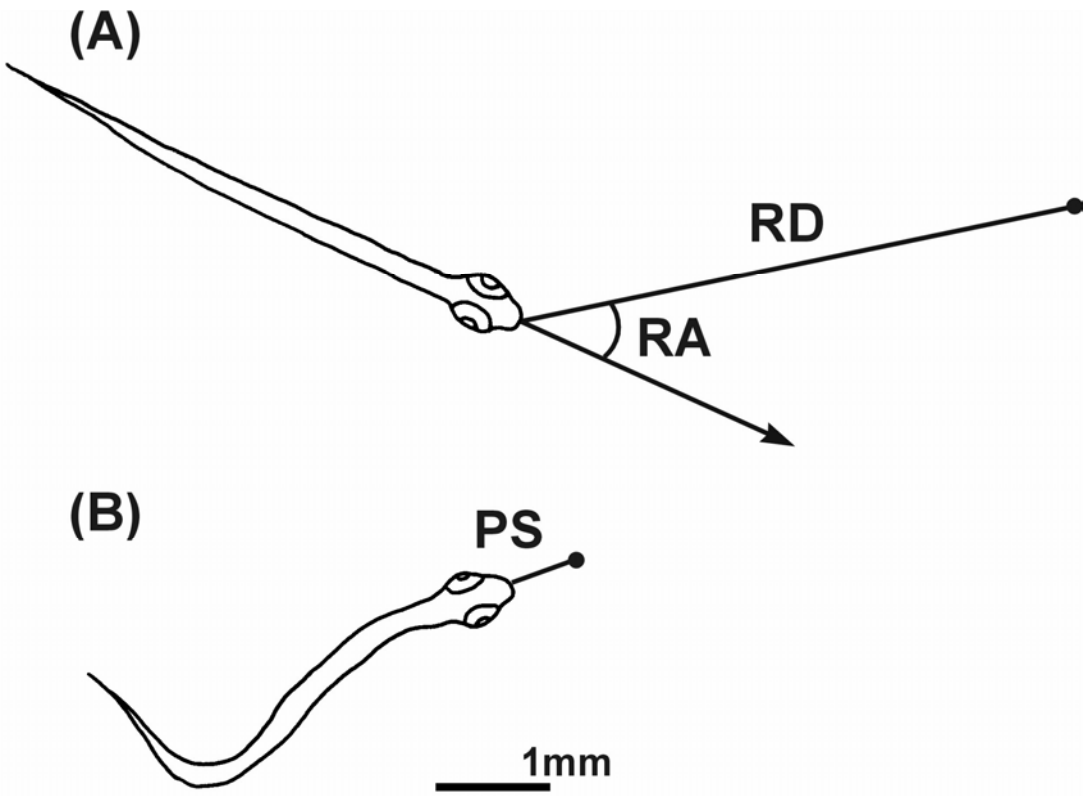

Figure 3.2. Diagrams of single frames from a feeding sequence of a striped trumpeter larva. Video analysis measurements were made in the frame (A) immediately before reacting to the rotifer prey, where the distance between the rotifer and the larva's rostral tip is the reactive distance (RD), and the angle between the forward direction of the larva and the rotifer position is the reactive angle (RA), and (B) after the larva has re-oriented to the prey detected in "A" and immediately before striking at the rotifer, where the distance between the larva's rostral tip and the rotifer is the pre-strike distance (PS). Scale bar indicates 1mm.

maximum RD observed would correspond to the maximum distance at which prey are identified (Browman *et al.*, 1990). In addition, the range of RD measured may in part reflect the underestimation error associated with horizontal orientation of the larvae, and the maximum RD in the worst case has the least error associated with it and in the best case is accurate. The rotifers added to the intensive culture were screened between 63 and 250 μm. A sample of rotifers was slowed with freshwater and 32 individuals were immediately measured with image analysis. The average dimensions of the rotifers (mean ± SE) were 302 ± 5 μm lorica length and 155 ± 4 μm lorica width. The length of egg bearing rotifers, including eggs, was 405 ± 9 μm (n = 6). Rotifer length and width were used to calculate a range of VA since the orientation and egg-bearing status of the rotifers detected by larvae could not be determined from video. For comparison with measured behavioural RD, equation 3 was solved for RD using MSA values calculated from histology as the visual angle and rotifer length (without eggs) as the prey size.

3.3.4 Statistics

The effects of larval age and retinal region on angular and linear cone cell densities, and MSA were tested with the Kruskal-Wallis non-parametric test (JMP® v.3, ©SAS Institute Inc.). The effect of retinal region on light path length within larval age was examined with one-way ANOVA, and Tukey-Kramer multiple comparison of means (P = 0.05) was used when the effect of retinal region was significant. The Kolmogorov-Smirnov two-sample test (two-tailed) (Sokal and Rohlf, 1995) was used to evaluate differences in the distribution frequency between filming days of RA and RD, grouped into 10° and 0.5mm classes, respectively.

3.4 Results

3.4.1 Larval growth

Growth data for cohorts 1 and 2 are provided in Chapter 2 (same cohort numbers), see section 2.4.1 and Fig.2.1, and increases in eye and lens diameters with age of cohort 1 larvae are given in Fig.2.13. Larvae from cohort 3, used to film feeding behaviour, hatched at 3.62 ± 0.02mm SL, and had reached 4.91 ± 0.03mm SL by first-feeding, day 8 post-hatching. Larval lengths on the days feeding behaviour was filmed were 5.24 ± 0.08mm SL on day 13, and 5.43 ± 0.07mm SL on day 17 post-hatching. Dorso-ventral eye diameters increased with age and were 292 ± 7, 331 ± 10, and 359 ± 12 μm on days 8, 13, and 17, respectively.

3.4.2 Retinal morphometry and theoretical acuity

There was a significant difference in the linear density of cone cells in striped trumpeter larvae between different regions of the retina (Kruskal-Wallis test, χ^2=23.76, df=8, P=0.0025) (Appendix 3, Table A3.2) but not between ages (Kruskal-Wallis test, χ^2=0.702, df=4, P=0.95) (Fig.3.3a) in the transverse histological sections. The highest densities were in the temporal, fundal, and dorso-temporal regions (39-41 cones.100 μm^{-1}), whilst the lowest were in the ventral regions (33-36 cones.100 μm^{-1}) (Appendix 3, Table A3.2). There was a decrease in the linear density of cones in sagittal sections from 47 ± 1 cones.100 μm^{-1} on day 7 to 43 ± 1 cones.100 μm^{-1} on day 26 post-hatching, but this was also not significant (Kruskal-Wallis test, χ^2=5.469, df=3, P=0.14) (Fig.3.3a). The corresponding angular density of cones increased significantly with larval age, in transverse sections from 0.147 ± 0.004 cones.$10'^{-1}$ visual arc on day 8 to 0.257 ± 0.007 cones.$10'^{-1}$ on day 21 post-hatching (Kruskal-Wallis test, χ^2=84.480, df=4, P<0.0001), and in sagittal sections from 0.165 ± 0.003 cones.$10'^{-1}$ visual arc on day 7 to 0.290 ± 0.007 cones.$10'^{-1}$ on day 26 post-hatching (Kruskal-Wallis test, χ^2=28.550, df=3, P<0.0001) (Fig.3.3a). There was

no effect of retinal region on the angular density of cones (Kruskal-Wallis test, χ^2=3.234, df=8, P=0.92) (Appendix 3, Table A3.3) nor on MSA (Kruskal-Wallis test, χ^2=3.191, df=8, P=0.92) (Fig.3.4; Appendix 3, Table A3.4) calculated from the transverse sections. Rod precursor nuclei were first observed in dorsal regions of the retina on day 12 (6.5mm SL, live) in sagittal sections and on day 14 (6.3mm SL, fixed) in transverse sections, and were apparent in ventral regions in older larvae (Fig.3.3b; Table 1; Appendix 3, Table A3.5). The angular density of rod precursor nuclei was higher in the dorsal compared with the ventral retina in larvae of all ages sectioned (Table 1; Appendix 3, Table A3.6). In contrast to cone density, both the linear and angular densities of rods increased with larval age in both transverse and sagittal sections (Fig.3.3b). More than one cone photoreceptor converged onto higher order ganglion cells, indicated by summation values greater than one, in larvae of all ages examined (Table 1).

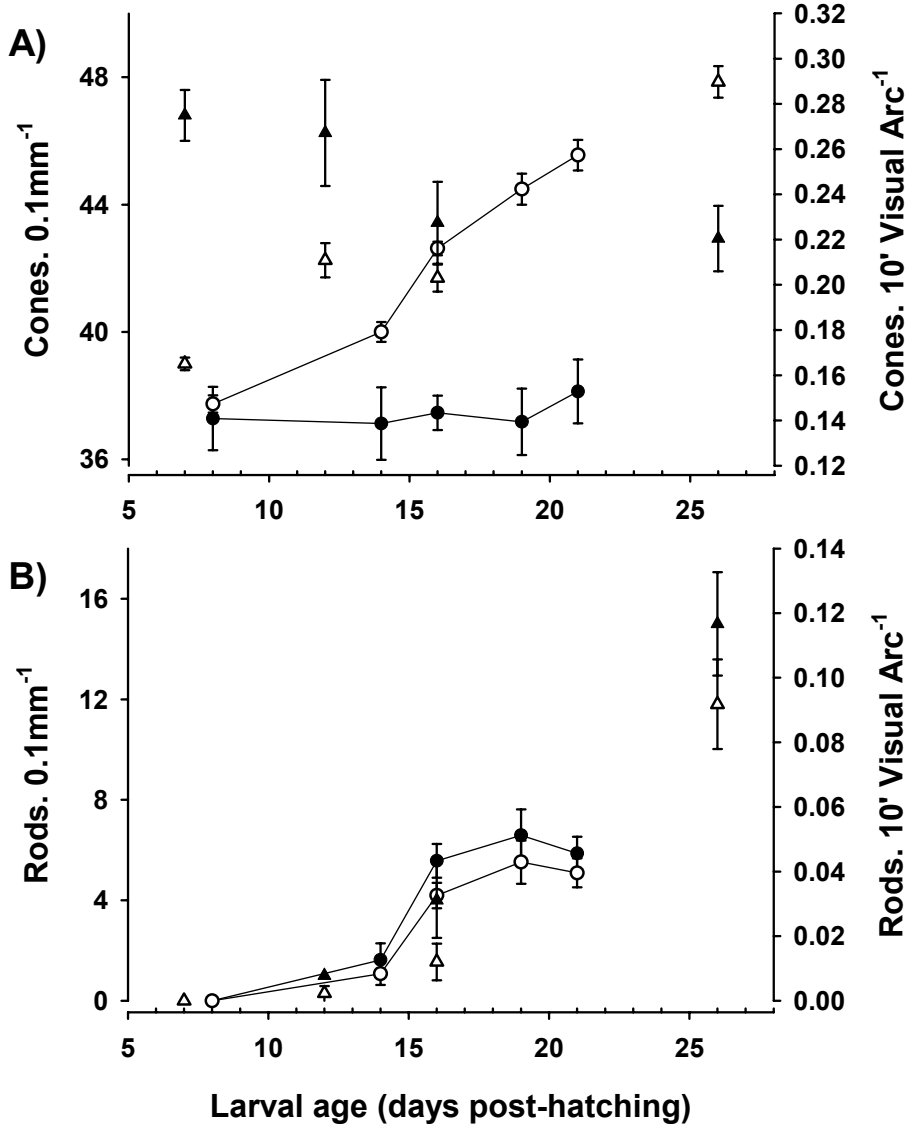

Figure 3.3. Changes in photoreceptor densities in the retinae of larval striped trumpeter, *Latris lineata*, with larval age. Linear density of photoreceptors in larvae observed by transverse sections (closed circles) and sagittal sections (closed triangles), and angular density of photoreceptors in transverse sections (open circles) and sagittal sections (open triangles). A) Cones and B) presumptive rod photoreceptors. Data points are pooled from replicate counts in different regions of the retina and from the larvae examined on each day. Values are mean ± SE, n = 5 to 37 (between 1 and 3 larvae per day, and 5 to 17 counts per larva, where count number is dependent upon eye size and distortion in retinal regions).

Table 3.1. Ratio of rod precursor nuclei in the dorsal and ventral retina and summation of photoreceptor nuclei to ganglion cells (GC) in striped trumpeter larvae with age in A, transverse and B, sagittal sections. Values calculated from angular cell densities, given as ratios or mean ± SE.

A: Transverse sections					
Age (days)	**8**	**14**	**16**	**19**	**21**
Ratio rod nuclei					
dorsal:ventral	0.0:0.0	0.02:0.00	1.5:1.0	3.5:1.0	1.8:1.0
Summation					
Cones/GC	1.4 ± 0.1	1.6 ± 0.1	1.3 ± 0.0	1.5 ± 0.1	1.5 ± 0.0
Rods/GC	0	0.08 ± 0.03	0.20 ± 0.03	0.27 ± 0.05	0.23 ± 0.02
PR/GC	1.4 ± 0.1	1.7 ± 0.1	1.5 ± 0.0	1.7 ± 0.1	1.8 ± 0.0
B: Sagittal sections					
Age (days)	**7**	**12**	**16**	**26**	
Ratio rod nuclei					
dorsal:ventral	0.0:0.0	0.01:0.00	0.02:0.00	2.0:1.0	
Summation					
Cones/GC	2.7 ± 0.3	1.9 ± 0.2	3.1 ± 0.3	1.9 ± 0.1	
Rods/GC	0	0.02 ± 0.02	0.19 ± 0.10	0.60 ± 0.09	
PR/GC	2.7 ± 0.3	2.0 ± 0.2	3.3 ± 0.3	2.5 ± 0.2	

PR = photoreceptor nuclei (cones plus presumptive rod nuclei).

The light path length was significantly greater in the dorsal compared with the ventral regions of the retina within the ages of larvae examined by transverse sections ($P < 0.05$) (Fig.3.4). The same pattern was found in sagittal sections (Fig.3.5), where the dorsal, dorso-temporal and temporal regions had the longest light path lengths.

The relative thickness of retinal layers changed with larval age, such that the IPL and light path length (PE + OS) increased while the GCL and INL decreased in dorsal, medial, and ventral areas of retina (Fig.3.6). Also, the relative thickness of the light path length was higher in dorsal and medial regions compared with the ventral retina.

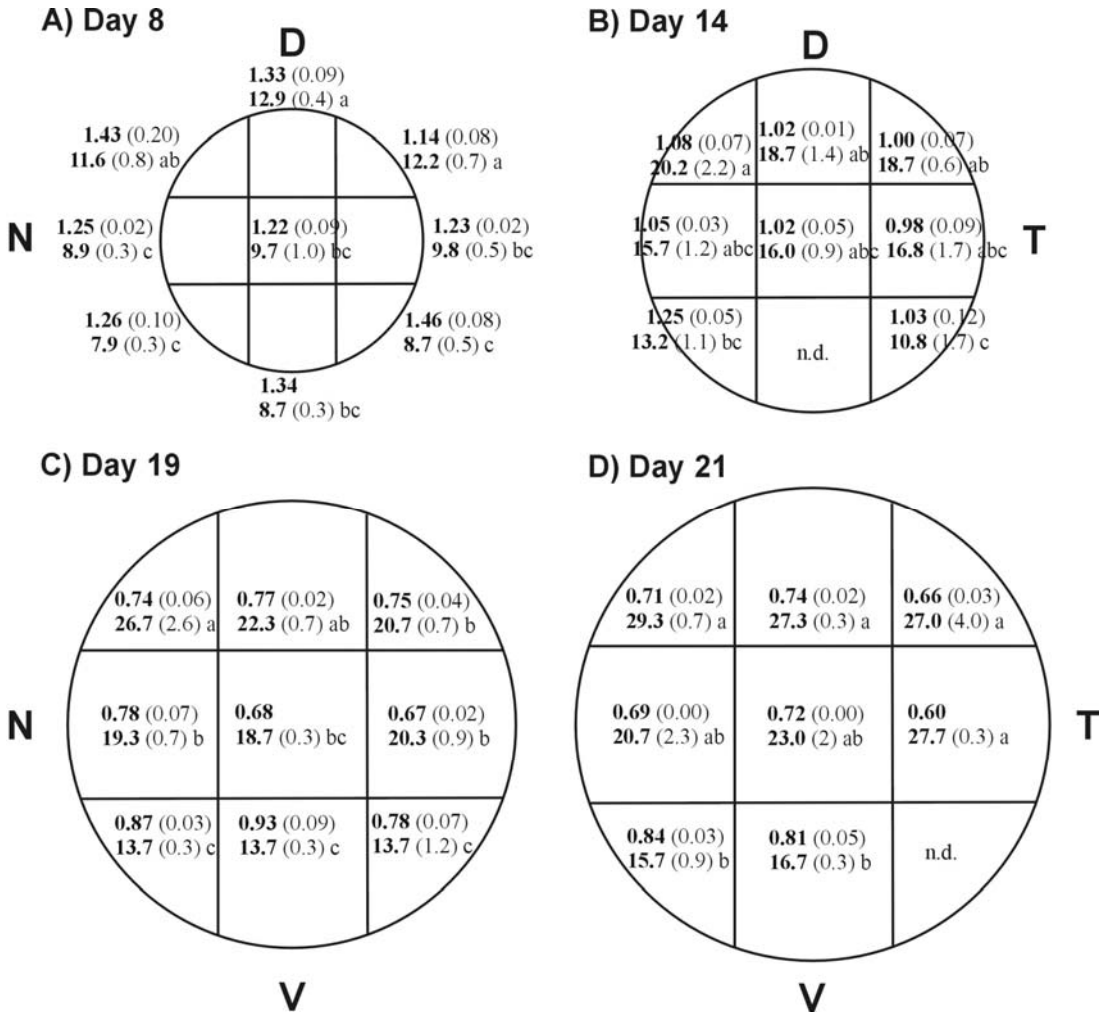

Figure 3.4. Minimal separable angles (MSA) and light path lengths (LP) in different regions of the retina in larval striped trumpeter, *Latris lineata,* determined from transverse sections. Circles are drawn proportional to eye diameter at each age. Top value in bold font in each region is MSA (°), bottom value in bold font is LP (μm), values are mean (SE). Different letters, a, b, and c denote significant differences (P=0.05) between light path lengths in different regions within each day. A) Day 8, standard length fixed (SL) = 4.81 ± 0.07mm, eye diameter (ED) = 311 ± 8 μm (values from 3 fish), B) Day 14, SL = 6.21 ± 0.17mm, ED = 406 ± 50 μm (values from 2 fish), C) Day 19, SL = 7.15mm, ED = 532 μm (values from 1 fish), and D) Day 21, SL = 6.95mm, ED = 562 μm (values from 1 fish). Abbreviations D, dorsal; N, nasal; T, temporal; V, ventral. n.d. indicates regions where values were not determined due to the presence of the embryonic fissure.

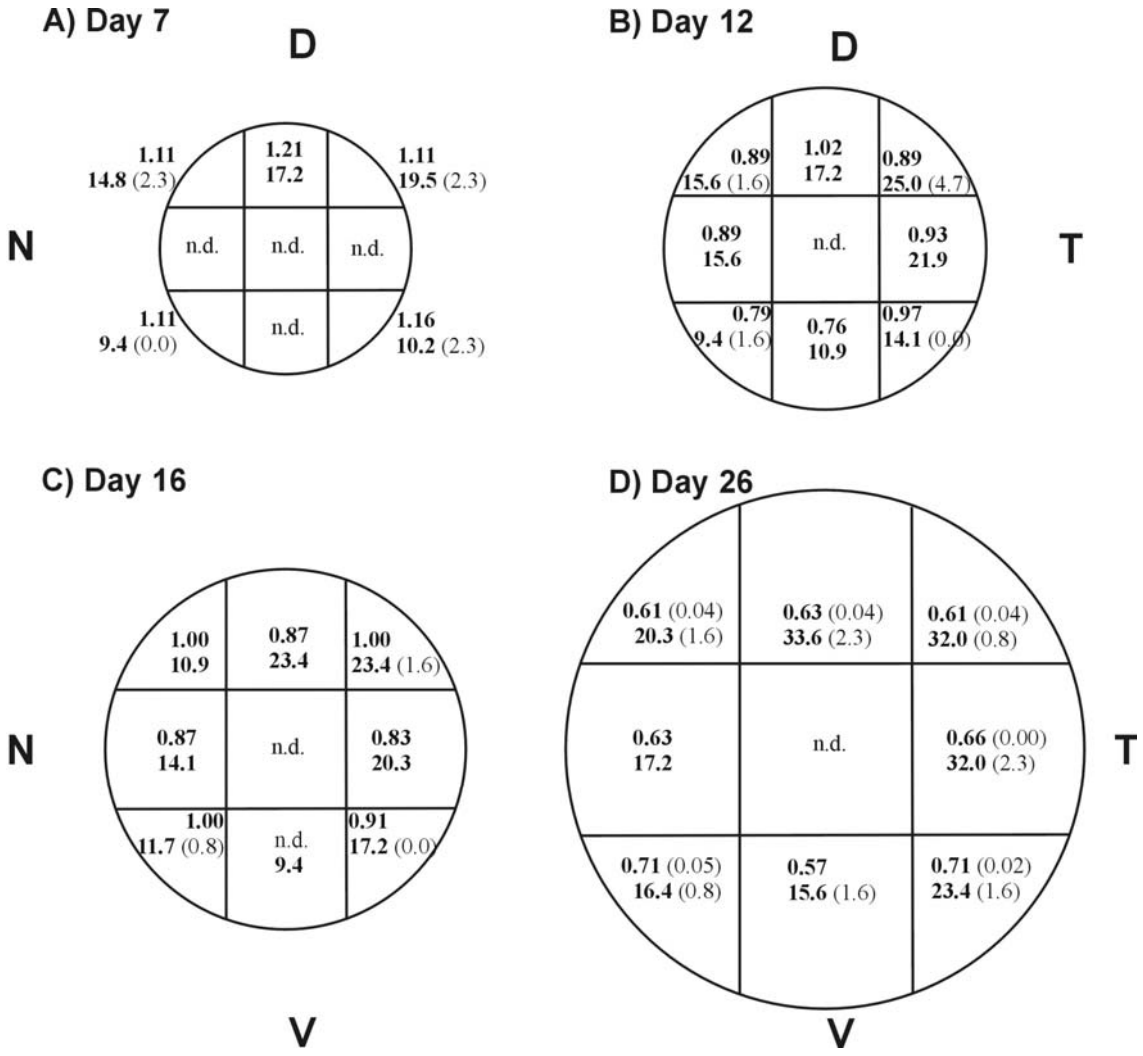

Figure 3.5. Minimal separable angles (MSA) and light path lengths (LP) in different regions of the retina in larval striped trumpeter, *Latris lineata,* determined from sagittal sections. Circles are drawn proportional to eye diameter at each age. Top value in bold font in each region is MSA (°), bottom value in bold font is LP (μm), values are individual measures or mean (SE) from a single fish on each day. A) Day 7, standard length live (SL) = 5.46mm, eye diameter (ED) = 288 μm, B) Day 12, SL = 6.52mm, ED = 369 μm, C) Day 16, SL = 7.23mm, ED = 413 μm, and D) Day 26, SL = 9.08mm, ED = 594 μm. Abbreviations D, dorsal; N, nasal; T, temporal; V, ventral. n.d. indicates regions where values were not determined due to the presence of the embryonic fissure or regions not represented in the sagittal sections.

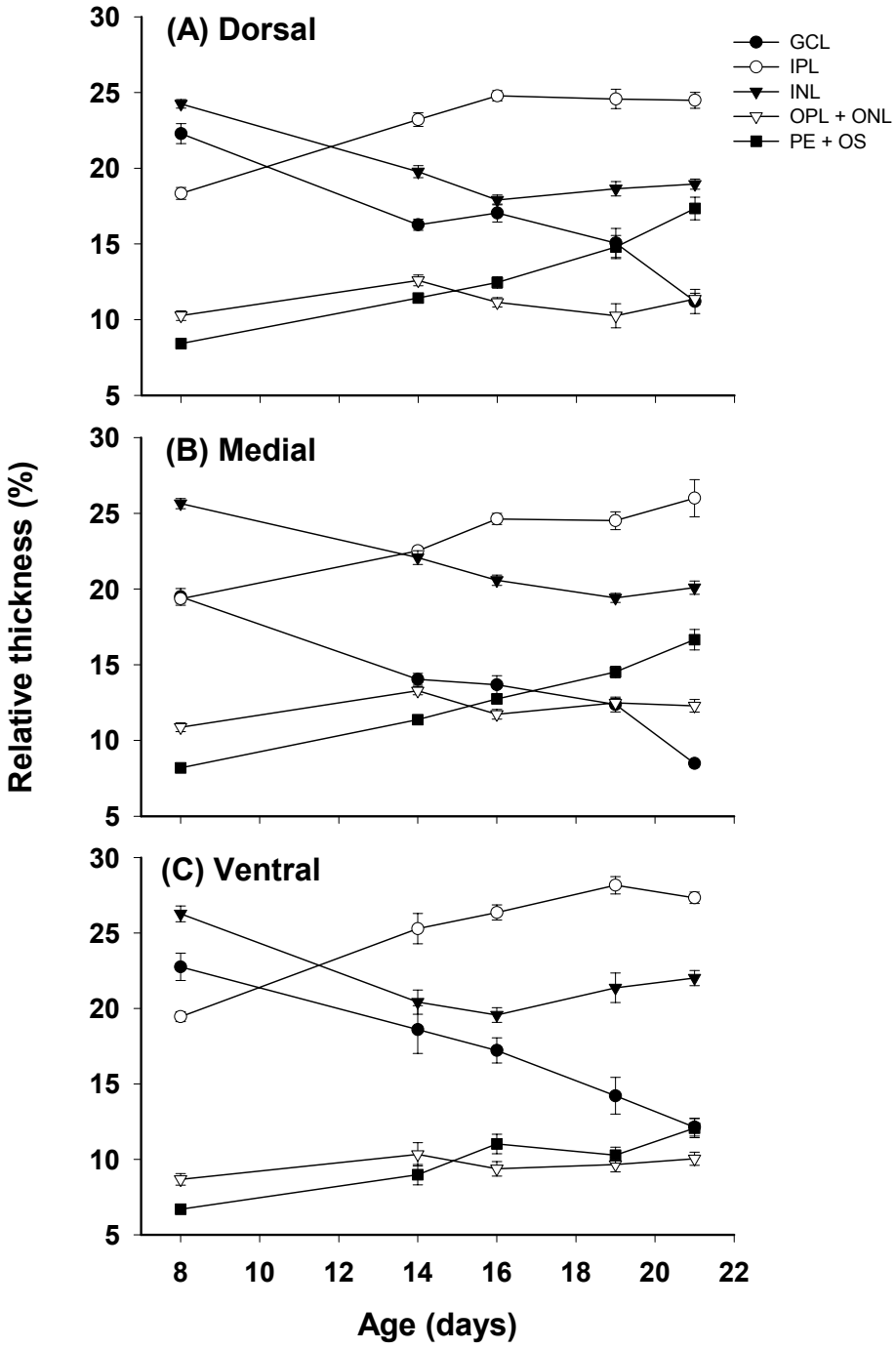

Figure 3.6. Relative thickness of layers in the (A) dorsal, (B) medial, and (C) ventral retina of larval striped trumpeter during ontogeny measured from transverse sections. Closed circles - ganglion cell layers, GCL; open circles - inner plexiform layer, IPL; closed triangles - inner nuclear layer, INL; open triangles - outer plexiform and outer nuclear layers, OPL+ONL; and closed squares - pigment epithelium and photoreceptor outer segment layers, PE+OS (= light path length). Values are mean ± SE from between 1 and 3 fish per day, with 3 to 9 replicates per dorsal, medial, and ventral area per fish.

3.4.3 Behavioural acuity

Striped trumpeter larvae have a move and stop mode of feeding, in which larvae swim, stop and scan within their visual field, and then either react to an object within the visual field or repeat the swim, stop and search behaviour. After locating a potential prey item, larvae turned their head toward the prey and swam toward it. As a larva came close to its prey, its body formed an S-posture, which in most cases was followed by a rapid forward strike at the prey. Larvae were often seen moving their head from side-to-side as they approached their prey or as they held the S-posture.

Eighteen feeding sequences on day 13 and fifty one sequences on day 17, were used in the analysis of larval striped trumpeter feeding behaviour. (Note: Not all of the feeding sequences yielded both RD and RA, and PS due to movement of the larvae out of the camera's field of view. Thus, the n values in captions for Figs.3.6&3.7 do not correspond to the total number of sequences analysed.) The proportion of feeding sequences resulting in capture of the rotifer prey was higher on day 13 than on day 17 post-hatching, 87% and 53%, respectively. This coincided with an increase in the proportion of sequences resulting in larvae forming an S-posture but turning away before striking from 7% on day 13 to 35% on day 17. Of the sequences finishing with a strike at a rotifer prey, prey were successfully captured on 93% of occasions on day 13 (n=13) and 82% of occasions on day 17 post-hatching (n=23).

On day 13 post-hatching, RD ranged from 1.3 to 5.07mm, with a mean of 2.60 ± 0.24mm (mean ± SE, n = 17) (Fig.3.7a), and on day 17 post-hatching RD ranged from 0.61 to 5.25mm, with a mean of 2.96 ± 0.21mm (n = 34) (Fig.3.7b). The maximum RD was 97% of larval SL on both of the days feeding behaviour was filmed.

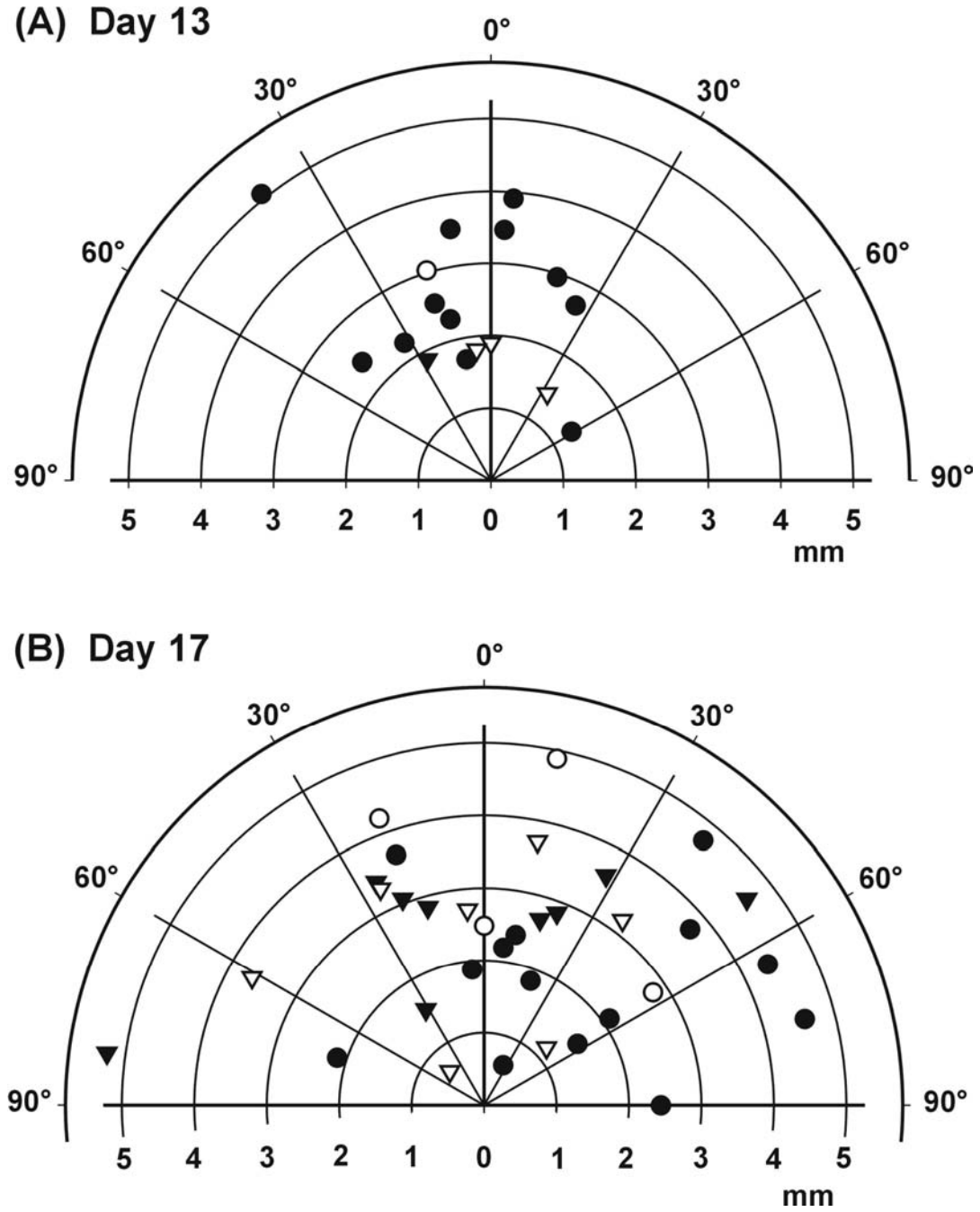

Figure 3.7. Reactive distances and angles: the position of rotifer prey relative to striped trumpeter larvae when the larvae first reacted to them on (A) day 13 post-hatching (n = 17), and (B) day 17 post-hatching (n = 34). The origin indicates the rostral tip of each larva. Larval standard lengths were 5.24 ± 0.08mm on day 13 and 5.43 ± 0.07mm on day 17 (mean ± SE, n = 10). Each data point is from one feeding sequence from one fish, and different symbols indicate the final outcome of that feeding sequence: closed circle, prey caught; open circle, prey missed; closed triangle, larva turned away before strike; open triangle, strike outcome uncertain.

On day 13 and day 17 post-hatching, mean PS calculated from completed sequences was similar on both days, measuring 0.44 ± 0.02mm (n = 17) and 0.46 ± 0.02mm (n = 36) respectively, equivalent to 8% of larval SL. Likewise, PS on day 13 post-hatching in the only incomplete sequence where a larva turned away before striking at its prey (0.48mm) was within the range of PS measured in completed sequences resulting in a strike at a rotifer (0.24-0.57mm). On day 17 post-hatching, the range of PS in incomplete sequences (0.23-1.18mm, mean 0.58 ± 0.07mm, n = 15) was greater than in completed sequences (0.31-0.72mm).

The RA of larvae to rotifer prey increased from 21 ± 7° (angular mean of pooled left and right-hand-side reactions ± 95% confidence interval; Batschelet, 1981) with a range of 0 to 58.8° on day 13 post-hatching, to 35 ± 8° with a range of 0 to 90.1° on day 17 post-hatching (Fig.3.7). There were no significant differences in the frequency distributions of RA (Fig.3.8a&b; Kolmogorov-Smirnov test, absolute value or 'unsigned' difference $D=0.29 < D_{0.05}=0.40$, $P=0.05$) or RD (Fig.3.8c&d; Kolmogorov-Smirnov test, unsigned difference $D=0.18 < D_{0.05}=0.40$, $P=0.05$) between days 13 and 17 post-hatching. However, an increase in the visual field was indicated by a greater range of RA observed, with similar maximum RD in each 10° sector of RA, on day 17 compared with day 13 (Fig.3.9).

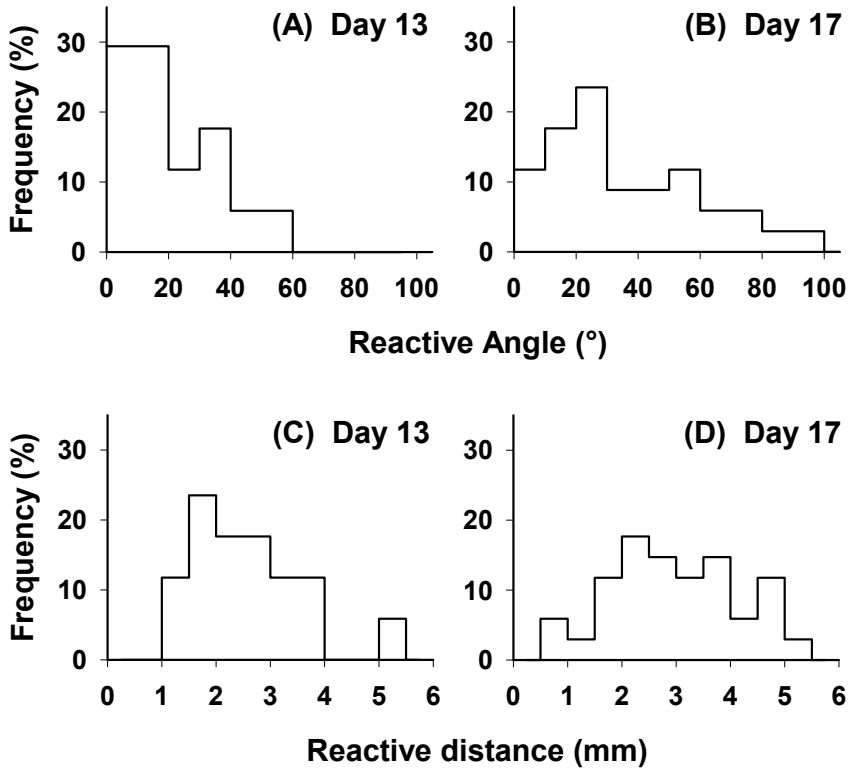

Figure 3.8. Frequency distribution of reactive angles (A&B) and reactive distances (C&D) of striped trumpeter larvae feeding on rotifers on day 13 (A&C; n = 17) and day 17 (B&D; n = 34) post-hatching.

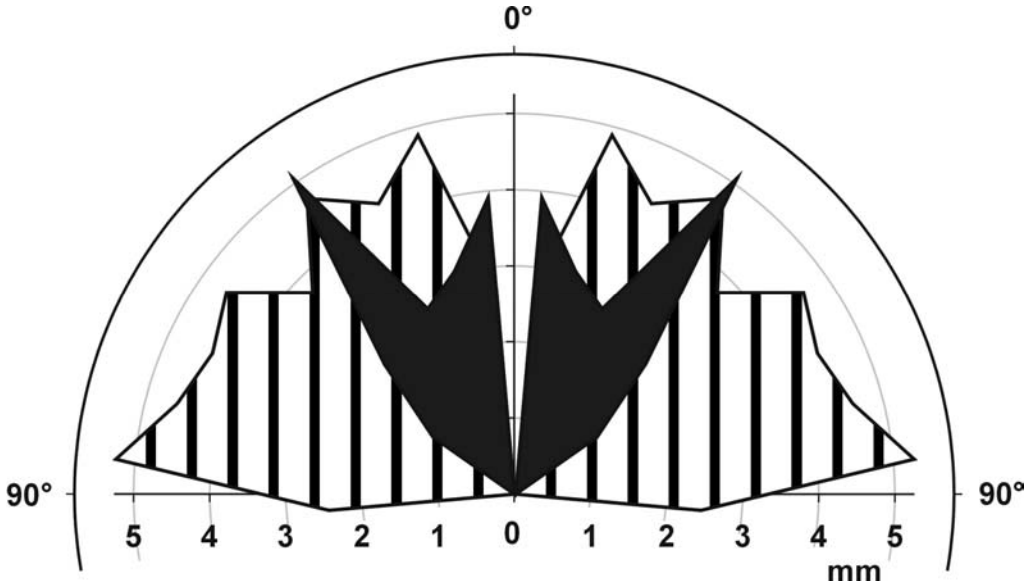

Figure 3.9. The horizontal visual field of striped trumpeter larvae on day 13 (black fill) and day 17 (striped fill) post-hatching, determined from maximum reactive distances in each 10° division of reactive angle from pooled left and right-hand-side reactions to rotifer prey. Nine feeding sequences contributed maximum reactive distances on day 13, and ten sequences on day 17.

3.4.4 Comparison of theoretical and behavioural acuity

The visual acuity of striped trumpeter larvae determined from feeding behaviour was 3°25' and 3°18'on days 13 and 17 post-hatching, respectively, when maximum RD and rotifer length (without eggs) were used in the calculation (Fig.3.10). The estimated acuity was halved when rotifer width was used in the calculation. In contrast, the estimate of acuity increased approximately eleven times when PS was used in the calculation rather than maximum RD. In comparison, the theoretical acuities determined from transverse sections were much lower; MSA decreased significantly with age from 1°17' MSA at day 8 post-hatching (coincident with first-feeding) to 44' MSA at day 21 post-hatching (Kruskal-Wallis test, χ^2=84.9, df=4, P<0.0001) (Fig.3.10). There was a similar decrease in MSA with age calculated from sagittal sections, reaching 39'at day

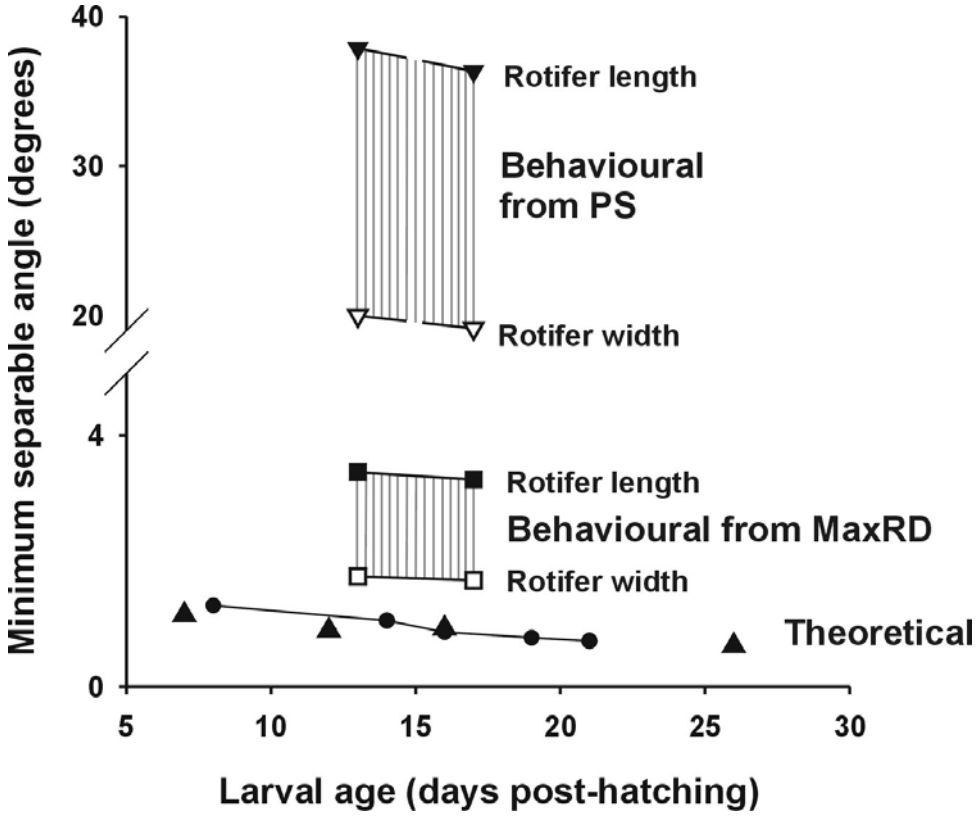

Figure 3.10. Improvement of theoretically determined visual acuity (minimum separable angle) in striped trumpeter larvae with ontogeny (transverse sections – closed circles, sagittal sections – closed upward triangles), compared with ranges of functional visual angles derived from feeding behaviour and calculated with maximum reactive distances (MaxRD), pre-strike distances (PS) and rotifer dimensions (302 μm length and 155 μm width).

26 post-hatching. Theoretical reactive distances to prey, derived from the theoretical values of MSA and prey size equivalent to the rotifer length in this study (302 μm, without eggs), were 13mm on day 8 increasing to 27mm on day 26 post-hatching.

3.5 Discussion

Striped trumpeter larvae follow the feeding pattern of a typical saltatory searching planktivore, swimming then stopping briefly to scan throughout their visual field for prey (Browman *et al.*, 1990). The range of RD measured in the present study reflects the larvae's use of their entire visual field for prey detection. Following initial prey detection (RD), planktivores display behaviours enabling them to precisely locate their prey prior to a strike. While approaching the detected prey and prior to adopting the pre-strike posture, striped trumpeter larvae were frequently observed moving their head from side-to-side. Rosenthal and Hempel (1970) suggested the scanned visual field of larval herring (*Clupea harengus*) is increased by lateral movements of the head while swimming. Possibly of greater significance, this side-to-side head movement, known as 'peering', has been identified as a mechanism to enhance distance discrimination in praying mantis larvae (Poteser and Kral, 1995), and as such would play a vital role in the ability of striped trumpeter larvae to precisely locate the prey item and time the strike. Browman *et al.* (1990) indicated that the binocular field and depth perception of white crappie (*Pomoxis annularis*) is likely enhanced by the nasal rotation of the eyes; however this was not examined in striped trumpeter.

As a result of the observation of peering associated with PS behaviour in striped trumpeter larvae, PS rather than maximum RD may better represent the position at which prey are clearly resolved. RD may indicate detection of prey motion and crude 'form' discrimination rather than acute prey resolution that follows during the PS stage. If so, this indicates that the functional acuity of larval striped trumpeter may be very poor indeed, exemplified by PS distances of just 0.44 and 0.46mm for larvae 13 and 17 days of age, respectively. This highlights the importance of providing sufficient prey density in larval culture to maximise

the likelihood of prey encounter and detection. In addition, larval prey detection in culture may be optimised by maximising prey size within the constraints of larval gape, manipulating prey contrast via prey diet (Dendrinos *et al.*, 1984) or light conditions (Utne-Palm, 1999), and providing suitable prey types (ie. not likely to move quickly or erratically out of the larva's visual range). Given the small visual range of larvae, models of larval growth and survival in the wild indicate the necessity for larvae to locate prey patches of relatively high density to facilitate successful feeding (Hunter, 1972).

The highest visual angle calculated in striped trumpeter larvae from PS distance, 38°, is comparable to values determined from the optokinetic responses of some pre-feeding yolksac larvae (Neave, 1984; Helvik and Karlsen, 1996) and other first-feeding larvae (Pankhurst *et al.*, 1993; Pankhurst, 1994) (Table 3.2). However, other studies using optokinetic responses or RD to prey report smaller visual angles in first-feeding larvae, between 2 and 7° (Table 3.2), comparable with the visual angles determined from maximum RD in striped trumpeter larvae. Neave (1984) suggested the optokinetic response of larvae may be in response to the movement of the lines used in this method, rather than a distinguishing between the lines, and thus the method may not be measuring the resolving ability of the larvae (Douglas and Hawryshyn, 1990). The use of larval RD to prey in assessing acuity is also confounded by factors which affect RD, including prey size (Confer *et al.*, 1978; Evans and O'Brien, 1988; Wanzenböck and Schiemer, 1989), visible prey size (Wright and O'Brien, 1982), prey movement (Wright and O'Brien, 1982; Utne-Palm, 1999), light intensity (reviewed in Douglas and Hawryshyn, 1990), turbidity (Vinyard and O'Brien, 1976), and larval search strategy (either 'saltatory' or 'cruise', Browman and O'Brien, 1992). In studies where visual angles are calculated from RD and a known prey size, the angles are similar for different prey sizes, since large prey are responded to at a greater distance (e.g. Breck and Gitter, 1983; Wanzenböck and Schiemer, 1989; Wanzenböck *et al.*, 1996) (Table 3.2). Both behavioural techniques provide a relative measure of larval abilities and the improvement of

Table 3.2. Behavioural visual acuities in larval and juvenile fishes, expressed as minimum separable angles, determined from different methods: A, optokinetic response and B, reactive distance to prey.

Species **Age/Developmental Stage/Size**	**Minimum Separable Angle**	**Author(s)**
Method A: Optokinetic Response		
Pleuronectes platessa, plaice		(Neave, 1984)
Early stage 1 (c.day 1)	c.42°	
First-feeding (late stage 1, c.day 12)	6-7°	
Pre-metamorphosis (stage 3b, c.day 43)	22’	
Late metamorphosis (stage 4b, c.day 60)	11’	
Scophthalmus maximus, turbot		(Neave, 1984)
Stage 1 (c.day 3)	c.21°	
First-feeding (late stage 1, c.day 4)	6-7°	
Late metamorphosis (stage 4b, c.day 30)	22’	
Forsterygion varium, triplefin		(Pankhurst *et al.*, 1993)
day 1 (first-feeding), c.5.4mm SL	28° 36’	
day 14, c.7.5mm SL	4° 18’	
Pagrus auratus, New Zealand snapper		(Pankhurst, 1994)
day 4 (first-feeding), 3.2mm SL	38°	
day 5	c.24°	
day 16, 4.7mm SL	8° 8’	
Hippoglossus hippoglossus, Atlantic halibut		(Helvik and Karlsen, 1996)
150 day°, c.6.5mm	25°	
200 day°	<5°	
225 day° c.11.5mm (first-feeding)	2-5°	
Method B: Direct measure of reactive distance to prey		
Lepomis macrochirus, bluegill		(Breck and Gitter, 1983)*****
27mm SL	c.42’	Prey:
43mm	c.30’	*Daphnia galeata* 1.04mm, *D. magna*
152mm	c.17’	2.64 and 2.59mm
Alburnus alburnus, bleak		(Wanzenböck and Schiemer, 1989)*****
8mm	320’	Prey:
12mm	150’	*Ceriodaphnia reticulata* 0.49 and
48mm	50’	0.65mm
Abramis ballerus, blue bream		(Wanzenböck and Schiemer, 1989)*****
9mm	270’	Prey:
14mm	200’	*C. reticulata* 0.49 and 0.65mm
51mm	60’	
Rutilus rutilus, roach		(Wanzenböck and Schiemer, 1989) *****
8mm	350’	Prey:
12mm	250’	*C. reticulata* 0.49 and 0.65mm
48mm	100’	
Premnas biaculeatus, maroon anemonefish		(Job and Bellwood, 1996)
day 3, 4.57mm	320’ (5°20’)	Prey:
day 10, 4.76mm	205’ (3°25’)	rotifer, 0.167mm length
Perca flavescens, yellow perch		(Wanzenböck *et al.*, 1996) *****
6.5mm SL	c.300’ (5°)	Prey:
25mm SL	c.60’ (1°)	*Artemia* nauplii 0.4mm,
		Ceriodaphnia sp. 0.49 and 0.65mm
Latris lineata, striped trumpeter		This study
day 13, 5.24mm SL	3°25’ (maxRD)	Prey:
	38° (PS)	rotifer, 0.302mm length
day 17, 5.43mm SL	3°18’ (maxRD)	
	36° (PS)	

MaxRD = maximum reactive distance of larvae to prey. PS = pre-strike distance.
***** No significant effect of prey size or prey type on calculated visual acuity.

functional acuity with age and fish size. However, comparison between the visual angles of species is complicated by the environmental factors used in different studies. It is clear that there is a rapid improvement in behaviourally determined MSA with ontogeny, but as the behaviour of striped trumpeter larvae indicates, the actual visual angle where an image is clearly resolved is uncertain.

Non-visual senses were likely involved in the feeding of striped trumpeter larvae in this study, and may have affected RD to prey. Vision is the primary sense involved in the feeding behaviour of striped trumpeter larvae, however non-visual senses are involved to a lesser degree (Chapter 2). Mechanoreception elicits reactions from mottled sculpin (*Cottus bairdi*) larvae at close distances (<0.5mm) and in any direction relative to neuromasts on the head and body (Jones and Janssen, 1992). Chemoreception initiates generalised feeding activity responses in larvae (Dempsey, 1978; Knutsen, 1992), however would be unlikely to produce a directional response to individual prey in the context of a culture tank where water flow mixes the water column containing a high density of prey. All larvae observed in this study reacted in a forward directed field and at greater distances than those for mechanoreceptors suggesting vision was the major sense involved in prey detection and as such, the RDs measured were attributed to larval visual capabilities.

Vision in fish larvae is constrained by the optics of small eye size, which dictates that images are projected onto relatively few photoreceptors in the retina. Acuity is maximised within the constraints of small eye size through the tight packing of small single cone photoreceptors in the retina (Kotrschal *et al.*, 1990; Pankhurst *et al.*, 1993; Pankhurst and Butler, 1996; Pankhurst and Eagar, 1996), as seen in striped trumpeter (Pankhurst and Hilder, 1998; this study). As eye size increases, existing cones enlarge (Blaxter and Jones, 1967; Pankhurst *et al.*, 1993; van der Meer, 1993; Pankhurst and Eagar, 1996; Pankhurst and Hilder, 1998), the retina stretches to increase cone spacing and new cones are added only at the retinal margins (Johns, 1981; Fernald, 1985). As a result linear cone densities decrease with increasing age, whereas angular densities increase as a result of increasing eye and lens size. This is exemplified in the present

study by an approximate doubling of the angular density of cones in larvae from first-feeding to day 26 post-hatching. The linear density of cones decreased in the cohort 2 larvae examined by sagittal sections, while there was little change in the linear density of cones in the transverse sections from cohort 1 larvae. This was likely reflecting more rapid cone enlargement and retinal expansion in cohort 2 larvae, which also showed slightly better growth than cohort 1 (see Chapter 2, Fig.2.1). In contrast with cones, rods are added throughout the retina during eye growth and increase scotopic sensitivity (Fernald, 1985), resulting in the concurrent increase in the linear and angular densities of rods in striped trumpeter.

The dorsal, dorso-temporal and temporal regions of the retina of striped trumpeter larvae were suited to acute image formation, indicated by the morphology of the retinal layers and photoreceptor cell density, which corresponds to a forward and downward directed theoretical visual field. The linear density of cone cells was lower in the ventral retina than in the temporal region, such that fewer cones would sample the image of an object in the ventral region, reducing resolution, compared with the temporal retina. Kawamura *et al.* (1984) used the ratio of cone cell density in the temporal and dorso-temporal regions to density in the ventral retina of red sea bream (*Pagrus major*) to demonstrate a shift in the region specialised for acute image formation from the temporal to the dorso-temporal at 30mm total length. This morphological shift was correlated with a behavioural shift in diet in juveniles collected from the wild, from planktonic copepods to amphipods, such that the forward directed visual field in fish <30mm was appropriate for planktivory, while the forward and downward directed visual field in fish >30mm was suited to benthic foraging (Kawamura *et al.*, 1984). An *area temporalis*, specialised for acute image formation and comparable to an area described in northern anchovy larvae (O'Connell, 1981), was identified in the temporal retina of striped trumpeter (Chapter 2), likely enhancing image formation in the forward-directed visual field. The earlier appearance of presumptive rod nuclei and the higher number of rods in the dorsal compared with the ventral retina in striped trumpeter larvae, indicate the dorsal retina is better equipped for scotopic (low

light) vision. In addition, the distance available for the capture of image forming light, the light path length (Pankhurst, 1989), was significantly higher in the dorsal than in the ventral retina. Another factor impacting upon the capacity of acute image formation in the ventro-nasal retina was the extensive insertion of the embryonic fissure into that region (Chapter 2) reducing the area covered by photoreceptors and likely creating a "blind spot".

Larvae possessing a visual axis in the direction of the mouth are well-equipped to capture prey, particularly as they position themselves very close to prey items before striking as seen in striped trumpeter. In adult fishes, where mechanoreception can play a major role in final strike trajectories (Janssen and Corcoran, 1993), the visual axis needs to be developed for optimal prey detection and predator avoidance at distance and not necessarily in the direction of the mouth. The dorso-temporal, temporal, and naso-temporal regions of the retina in adult fishes are often specialised for acute image formation (Tamura, 1957), where theoretical acuity is between 1 and 10 minutes of arc in pelagic fishes (Tamura and Wisby, 1963; Pankhurst, 1989). A marked ontogenetic improvement in MSA from larvae to adult is required to achieve this, and indeed has been identified in several studies (e.g. in New Zealand snapper from 2° at 4 days post-hatching to 3-4' visual arc in adults (Pankhurst and Eagar, 1996). Larval striped trumpeter demonstrated an improvement in MSA with age, but no pattern of regional specialisation in MSA was detected in the age classes examined. Thus, examination of older fish would be required to determine if and when regional specialisation occurs during development in striped trumpeter.

Behaviourally determined visual angles approach, but are often higher than theoretical values of acuity in adult fishes, such that functional acuity is close to but not as good as that predicted from the visual structures (Powers and Easter, 1983). However, this is not the case in larvae where values derived from behaviour may be much lower than theoretical MSA (Neave, 1984; Pankhurst *et al.*, 1993; Pankhurst, 1994; Helvik and Karlsen, 1996; present study). There are particularly rapid improvements in behavioural visual angle in the early larval stages, when concurrent changes in MSA are relatively small (e.g. Neave, 1984;

Pankhurst *et al.*, 1993; Pankhurst, 1994; Wanzenböck *et al.*, 1996). The discrepancy between functional and theoretical acuity, and the rate at which they improve have been discussed by several authors. The ongoing development of higher order neural pathways, and the processing of visual signals received by the eye may facilitate the improvement of functional acuity (Pankhurst *et al.*, 1993; Pankhurst, 1994). In addition, the MSA calculation of Neave (1984) assumes the minimum number of cones are involved in distinguishing two points, which can be related to theoretical prey detection, however prey recognition that elicits a behavioural reaction may require the involvement of a higher number of cones (Wahl *et al.*, 1993; Job and Bellwood, 1996). Indeed, it has been suggested that groups of four double cones and a central single within a square mosaic function as a single visual unit converging onto higher order cells (van der Meer and Anker, 1984; Browman *et al.*, 1990). Since the functional neural pathways are difficult to determine for inclusion in a model of MSA, the formula of Neave (1984) provides an optimistic estimate of larval capabilities, while functional acuity measured from behaviour is a better measure of larval competence (Wanzenböck *et al.*, 1996).

However, functional acuity must be carefully interpreted since calculated visual angles are indirectly influenced by environmental factors that affect functional RD in fishes. While the maximum RD measured in the present study were just under a larval standard length, which is comparable to other studies (Blaxter, 1986), different RD are likely to result from different environment conditions. Increasing prey size increases RD of planktivorous fishes (Confer *et al.*, 1978; Evans and O'Brien, 1988; Wanzenböck and Schiemer, 1989). Since the orientation of prey at the time of detection by striped trumpeter larvae in the present study was unknown a range of visual angles are presented, between values calculated with rotifer width and rotifer length. Hunter (1980) suggested prey width is the critical dimension for the ingestion of prey. If this is the case, then larvae should be able to determine prey width from at least PS distance to ensure strikes are made at prey of an appropriate size. Egg-bearing rotifers that have a greater total length and are a larger visual target may have contributed to a portion of the feeding sequences analysed, although this was not determined in

the present study. Other factors influencing RD are the visible size of prey (Wright and O'Brien, 1982), prey movement (Wright and O'Brien, 1982; Utne-Palm, 1999), and the light environment (Vinyard and O'Brien, 1976; Douglas and Hawryshyn, 1990; Utne, 1997; Utne-Palm, 1999). The light intensity used in this study, 2.3 $\mu mol.s^{-1}.m^{-2}$, was within the range for optimal feeding in striped trumpeter larvae (1-10 $\mu mol.s^{-1}.m^{-2}$), defined in terms of the proportion of the population feeding and larval feeding intensity (Chapter 4; Cobcroft *et al.*, 2001). The response of striped trumpeter larvae to a range of prey types and environmental conditions may be quantified using the technique developed in this study to investigate feeding behaviour in larvae. For example, microalgae-induced turbidity (greenwater) can variably increase or decrease feeding performance in larval striped trumpeter (Chapter 4; Cobcroft *et al.*, 2001), but the effect of turbidity on feeding behaviour remains to be measured.

The prey capture success of larval striped trumpeter declined with age, primarily due to an increase in the proportion of incomplete feeding sequences, but also due to a decrease in the proportion of strikes resulting in prey capture. In contrast, larval anchovy (*Engraulis mordax* - Hunter, 1972) and juvenile yellow perch (*Perca flavescens* - Mills *et al.*, 1984) displayed a rapid improvement in capture success with age. Improvements in aiming capabilities, and increases in larval speed and mouth gape have been associated with increases in prey capture success during ontogeny (Drost, 1987). Despite increases in capture success, feeding sequences in larval anchovy were terminated at all stages, most commonly after the initial orientation of the head to the prey (Hunter, 1972). In the sequences of striped trumpeter feeding, the most common point of termination was after formation of the strike S-posture. Hunter (1972) found the PS distance of larval anchovy to their prey was higher in incomplete than in completed sequences (0.81mm and 0.41mm, respectively), and suggested failure of the larvae to complete the sequence was due to their inability to closely approach prey. This was not the case in striped trumpeter, where PS was similar in complete and incomplete sequences, although the increased range of PS in incomplete sequences on day 17 suggests the larvae may have been unable to stabilise their position relative to the prey. The increase in the proportion of

incomplete sequences may also have been due to increased satiation levels in the older larvae, since searching and pursuit of prey continues despite the fact that feeding intensity declines as satiation is approached (Hunter, 1972). The incomplete sequences may also reflect increased prey selectivity in older larvae, such that rotifer size determined from PS was not appropriate, or that the rotifers themselves were no longer preferred.

Larval age, between days 13 and 17 post-hatching, did not affect the maximum RD determined in striped trumpeter larvae. This contrasts with other studies, where RD increased with fish size and age (Breck and Gitter, 1983; Wanzenböck and Schiemer, 1989; Browman and O'Brien, 1992). The increase in larval size in this study was minimal and may account for the similar RD measurements. In addition, satiation may have limited the RDs displayed, since reactive distance is maximal in fishes immediately following starvation when the motivation to detect prey is highest (Confer *et al.*, 1978). Filming in the present study was undertaken approximately one hour after the addition of live food to the culture tank, likely enough time for larvae to fill their guts (Hunter, 1972). Examination of feeding responses in a greater age and size range of larvae and using starved fish would provide a better indication of changes in the absolute capabilities of striped trumpeter. Nevertheless, the present study is useful in understanding feeding behaviour relative to culture situations where the influence of starvation is small.

Striped trumpeter demonstrated an increased visual field with larval age, due to the increased range of RA from day 13 to day 17 post-hatching used in prey location. A similar increase in RA was reported with age in larval golden shiner (*Notemigonus crysoleucas* - Browman and O'Brien, 1992) and larval *Premnas biaculeatus* (Job and Bellwood, 1996). The precise mechanism for the increase in RA in striped trumpeter was not determined. However, it may be due to increased mobility of the eyes in the head, rather than changes in the structure of the retina in different regions. Models of prey detection and search volume in larval fish utilise RD and RA in the horizontal plane extrapolating to three dimensions (e.g. Wanzenböck and Schiemer, 1989) and where available utilise

measurements in the vertical plane (e.g. Hunter, 1972). An increase in RA, such as that seen in striped trumpeter, may be responsible for significant increases in search volume, beyond those due to increases in RD only, such that estimated prey density requirements for larvae decline with age (Hunter, 1980). Hunter (1980) reported prey density requirements for laboratory reared larvae ranging from 1 to 4 prey (microcopepods) per ml. More detail of the nutritional requirements for growth and survival of striped trumpeter larvae are required before a model to predict prey requirements can be developed.

Striped trumpeter larvae have a relatively small functional visual field, in part limited by the development of the retina. Within the constraints of small eye size, the dorso-temporal retina is specialised for acute image formation and scotopic sensitivity. This corresponds well with the forward and laterally directed visual field observed in feeding larvae. The properties of the larval visual field under different environmental conditions, such as light intensity, turbidity, and prey type, and characterisation of the visual field in the vertical plane remain to be investigated.

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4. Chapter Four. The effects of light intensity and algae-induced turbidity on feeding behaviour of larval striped trumpeter

4.1 Abstract

Larvae of the striped trumpeter were used to examine feeding performance in relation to light intensity and algal cell-induced turbidity (greenwater) in short-term feeding experiments. Larvae reared in greenwater fed equally well in clearwater in a light intensity range of 1-10 $\mu mol.s^{-1}.m^{-2}$, when evaluated in terms of both the proportion of larvae feeding and larval feeding intensity. An ontogenetic improvement in photopic visual sensitivity of larvae was indicated by improved feeding at 0.1 $\mu mol.s^{-1}.m^{-2}$, from 26 ± 5% of larvae feeding and 0.027 ± 0.005 rotifers consumed per feeding larva per minute on day 8, to 96 ± 2% and 0.221 ± 0.007 rotifers $consumed.larva^{-1}.min^{-1}$ on day 23 post-hatching. Algal cell-induced turbidity was shown to reduce incident irradiance with depth, indicated by increasing coefficients of attenuation (1.4 to 33.1) with increasing cell densities (0 to 2 x 10^6 $cells.ml^{-1}$), although light intensities in the feeding experiment test chambers, at the algal cell densities tested, were within the optimal range for feeding (1-10 $\mu mol.s^{-1}.m^{-2}$). Algae-induced turbidity had different effects on larval feeding response dependent upon the previous visual environment of the larvae. Young larvae (day 9 post-hatching) reared in clearwater showed decreased feeding capabilities with increasing turbidity, from 98 ± 1% feeding and 0.153 ± 0.022 rotifers $consumed.larva^{-1}.min^{-1}$ in clearwater to 61 ± 10% feeding and 0.042 ± 0.004 rotifers $consumed.larva^{-1}.min^{-1}$ at 56 nephelometric turbidity units (NTU), while older clearwater reared larvae fed well at all turbidities tested. Likewise, greenwater reared larvae had increased feeding capabilities in the highest algal cell densities tested (32 and 66 NTU) compared with those in low algal cell density (6 NTU), and clearwater (0.7 NTU) to which they were naive. It is suggested that turbidity alters feeding response in larvae through its effect on prey visibility rather than through

changes in light intensity. The results of this study raise the possibility that the previous experience of either a clearwater or greenwater visual environment may affect subsequent feeding responses of larvae, and this is an hypothesis that requires further investigation.

Keywords: turbidity, light intensity, experience, greenwater, feeding, striped trumpeter, marine fish larvae

4.2 Introduction

Most marine fish larvae are primarily visual feeders (Blaxter, 1986), and as such the light regime of their environment is critical for optimal feeding. Light intensity, spectral quality, and turbidity are known to affect larval feeding capabilities by altering prey search behaviour, reactive distances, and ultimately feeding success (Batty, 1987; Lazzaro, 1987; Huse, 1994; Link and Edsall, 1996; Utne-Palm, 1999). The feeding responses of larvae in different light environments change with the developing visual capabilities of larvae (Blaxter, 1968; Blaxter, 1969), and have been correlated with larval distribution in the wild (Job and Bellwood, 2000) and used to determine appropriate light conditions for larval culture (Pankhurst and Hilder, 1998). In turbid media, a high level of scattered light occurs where particle size is greater than the light wavelength, light intensity is reduced with depth, and spectral quality may also be altered (Duntley, 1943; Lythgoe, 1988). Some authors have demonstrated feeding performance of fish will be adversely affected in turbid environments and suggested this would be the general case (Benfield and Minello, 1996; Utne, 1997), however other studies have produced conflicting results.

The use of algal cell-induced turbidity in larval culture, known in aquaculture as greenwater, has been shown to improve visual feeding responses of larvae, including the timing of first-feeding, the proportion of larvae feeding, and feeding intensity (Naas *et al.*, 1992; Lazo *et al.*, 2000). Inert particle-induced turbidity has also enhanced feeding performance of larvae (Bristow and Summerfelt, 1994), but in other studies larval feeding has been unaffected or has

declined with increasing turbidity (Johnston and Wildish, 1982; Breitburg, 1988; Gulbrandsen *et al.*, 1996). Miner and Stein (1993) postulated the interaction between surface light intensity and turbidity may account for the contrasting results in the literature. The latter authors suggested that at high surface light intensity prey visibility is enhanced with increasing turbidity, whereas at low surface light intensity, increasing turbidity reduces light intensity in the water column below the visual threshold for feeding of larvae and consequently feeding response declines. In order to test this theory, the visual threshold for feeding by the larvae, and the attenuated light environment in turbid water, must be known.

The aims of this study were to investigate the effect of light intensity on larval feeding performance, and to examine the influence of algal cell-induced turbidity on the feeding response of larvae, particularly taking into account the range of light intensities available in a greenwater environment. The species used in this study was the striped trumpeter *Latris lineata* (Schneider, 1801), a temperate marine fish, which is being assessed as a potential candidate for aquaculture at the University of Tasmania, Tasmanian Aquaculture and Fisheries Institute. Striped trumpeter larvae are primarily visual feeders (Pankhurst and Hilder, 1998), however very little is known of their biology in the wild (Furlani and Ruwald, 1999), and the optimal parameters for their culture are currently under investigation. Larvae were reared in either clearwater or greenwater and their feeding responses were tested in short-term trials.

4.3 Materials and Methods

4.3.1 Larval culture

Larvae were sourced from three cohorts (cohort 1 - April 2000, cohort 2 - September 1999, and cohort 3 - October 1999) for feeding trials. Gametes were hand-stripped from wild-caught, acclimated, broodstock held at the Tasmanian Aquaculture and Fisheries Institute, Marine Research Laboratories, Taroona

(Morehead *et al.*, 2000). Hatching occurred 5 days post-fertilisation at 14°C, and yolksac larvae were stocked into the rearing tanks 1-3 days post-hatching at a density of 43-50 larvae.ml^{-1}. Larvae were fed rotifers enriched with Tahitian *Isochrysis* sp. and DHA Selco® (INVE Aquaculture, Belgium), twice daily at 5.ml^{-1}, and wild harvested harpacticoid copepods were added every few days at 0.01-0.05.ml^{-1}. All cohorts were reared under a photoperiod of 14-h light/10-h dark and tanks were siphoned daily to remove mortalities.

Cohort 1 larvae were reared in a 3000-l square tank (200 x 200 x 90 cm) at 15-17°C in a greenwater culture with the green alga *Tetraselmis suecica* maintained at 0.088 ± 0.007 x 10^6 cells.ml^{-1} and a turbidity of 5.01 ± 0.23 NTU. Light intensity at the surface, provided by two Osram Biolux® fluorescent lights suspended 1 metre above the water, ranged from 2 μmol.s^{-1}.m^{-2} at the edges to 19 μmol.s^{-1}.m^{-2} in the centre (50-1000 lux). An incandescent light was used to fade in and out the light phase. Cohorts 2 and 3 were reared in clearwater and greenwater respectively, in 1000-l cylindroconical tanks, 150 cm diameter and 90 cm deep at the centre, with blue sides and base. Cohort 2 was reared in recirculating 0.2 μm filtered seawater at 15.0°C, with 20-25% water filtration per hour. Light intensity provided by a cool white fluorescent globe 1 metre above the water was 1.3 - 3.4 μmol.s^{-1}.m^{-2} (700 lux) at the surface. Cohort 3 was reared at 16-18°C with *T. suecica* at 0.5 x 10^6 cells.ml^{-1} and a turbidity of 3.5 NTU, maintained by daily algae exchanges of 10% of tank volume. A single incandescent globe suspended 1 metre above the water provided light intensity ranging from 0.02 μmol.s^{-1}.m^{-2} at the edges of the tank to 22 μmol.s^{-1}.m^{-2} in the centre (0.2-3000 lux).

Larval growth in cohort 1 was determined by measuring ten larvae per day. In cohorts 2 and 3, five larvae were measured on alternate days from days 4 to 20 post-hatching and twenty larvae were measured on experiment days. The standard length (SL) of larvae was measured with Scion ImagePC Beta 3b analysis software on whole larva images captured from a Wild M5 stereo microscope and a Sony CCD camera.

4.3.2 Light intensity and turbidity measurement

Light intensities were measured with a Li-Cor LI-250 light meter with an underwater flat Quantum sensor LI-192SA (calibrated to air or water depending on the medium) and a Profisix lux meter (Gossen). Turbidities were measured in nephelometric turbidity units (NTU) using a Hach 2100P portable turbidimeter.

4.3.3 Feeding experiments - general procedures

On the morning of each experiment, lights over the culture tanks remained switched off, which extended the dark period, and 20 larvae were siphoned from the culture to confirm gut evacuation prior to commencing the feeding trial. Approximately 22 larvae were then siphoned into replicate 3-l blue goblet-shaped test chambers, along with 500 ml of culture water. Filtered seawater (0.2 μm) and/or the alga *T. suecica* were added according to the treatment (described below), increasing the volume to 3-l. Test chambers were placed under the treatment lighting conditions, and rotifers were then added at a density of $2.ml^{-1}$, marking the commencement of the feeding duration (0.5, 1 or 2 h, see respective experiment methodologies below). Transfer and handling from the large culture tanks to the test chambers did not appear to affect larval feeding behaviour. This was indicated by observations of larvae feeding immediately after rotifers were added and by equivalent or better feeding responses of larvae in the control treatment in experiment 1 (10 $\mu mol.s^{-1}.m^{-2}$ light intensity in greenwater) compared to the clearwater treatment. As such, no acclimation period was used in any of the feeding experiments. Rotifers were fed *T. suecica* to standardise prey colour, before being used in the feeding trials. Addition of feed to individual replicate test chambers was staggered at ten minute intervals, providing time to assess feeding at the end of each replicate's feeding duration. In all experiments there were five replicates for each treatment. At the end of the feeding duration for each replicate, larvae were collected on a 100 μm screen, pipetted onto chilled microscope slides, squashed with a coverslip, and examined for evidence of feeding under a dissecting microscope. Individual rotifers were either identified whole or by mastax (mouthparts) only, and

counted. Striped trumpeter larvae have a straight, transparent gut that enables prey items to be readily identified in squash preparations. The proportion of larvae feeding was calculated for each replicate. In order to standardise the rate of food intake, accommodating differing feeding durations within experiment 2 and between experiments, feeding intensity in all treatment replicates was calculated as the number of rotifers consumed per feeding larva per minute.

4.3.4 Experiment 1 - The effect of light intensity under clearwater conditions on feeding performance of larvae reared in greenwater

Experiment 1 examined the effect of light intensity in clearwater conditions on the feeding performance of larvae reared in greenwater (cohort 1). The light intensity treatments tested, 0.1, 1, 5, and 10 $\mu mol.s^{-1}.m^{-2}$ at the test chamber surface (8, 50, 200, and 400 lux, respectively), were provided by Biolux® fluorescent tubes either uncovered or covered by layers of shade-cloth to achieve the required treatments. The shade-cloth did not change the spectral range of the light source, confirmed using a Li-Cor LI-1800 portable spectroradiometer to be between 400 and 700 nm (Appendix 2). Because cohort 1 larvae were reared in greenwater, addition of a greenwater treatment (10-GW), with similar algal cell density and turbidity to the larval culture tank and at the highest light intensity (10 $\mu mol.s^{-1}.m^{-2}$) provided at the water surface in clearwater treatments, was used to control for the transfer of larvae into a novel clearwater environment. The turbidities of the 10-GW and the clearwater treatments were 5.08 ± 0.31 and 0.69 ± 0.03 NTU, respectively. Feeding performance of larvae was assessed on days 8, 15, and 23 post-hatching, with a 1 h feeding duration.

4.3.5 Experiment 2 - The effect of algal cell-induced turbidity on the feeding performance of larvae reared in clearwater

The effect of algal cell-induced turbidity on feeding performance was tested with larvae reared in clearwater (cohort 2), thus without prior experience of this environment. The treatments used were clearwater (CW), and three densities of

the green alga *T. suecica*, 0.1, 0.5, and 1.0 x 10^6 cells.ml^{-1}, with resulting turbidities of 0.70 ± 0.12, 5.99 ± 0.82, 23.2 ± 2.1, and 55.9 ± 5.1 NTU (mean ± SE, n = 30), respectively. The light intensity at the tank surface was 9.22 ± 0.10 μmol.s^{-1}.m^{-2} (400 lux), provided by Biolux® fluorescent globes, and was above the feeding threshold for all ages of larvae examined in experiment 1. The feeding response of larvae was assessed on days 9, 16, and 23 post-hatching, with feeding durations of 2 h on days 9 and 16, and 0.5 h on day 23. In experiment 1, 23 day old larvae, mastax of rotifers were counted to determine prey ingestion rate because of the increased larval ingestion and digestion rates in older larvae. In experiment 2, the reduction in feeding duration on day 23 facilitated the counting of individual prey in the gut of these older larvae.

4.3.6 Experiment 3 - The effect of algal cell-induced turbidity on the feeding performance of larvae reared in greenwater

The effect of algal cell-induced turbidity on feeding performance was tested with larvae reared in greenwater (cohort 3), thus experienced with this environment. The same tank surface light intensity (9.22 ± 0.10 μmol.s^{-1}.m^{-2}) and algal-cell density treatments (CW, 0.1, 0.5, and 1.0 x 10^6 cells.ml^{-1}) were used as in experiment 2, with turbidities of 0.68 ± 0.08 NTU for the CW, and 5.73 ± 0.42, 31.7 ± 2.6, and 66.3 ± 2.7 NTU (mean ± SE, n = 20) for the increasing cell densities, respectively. Feeding response of larvae was assessed on days 10 and 15 post-hatching, with a 2 h feeding duration. Low survival of larvae precluded assessment of older larvae in this experiment.

4.3.7 Light attenuation and reflected light profiles within the test chambers with increasing algal cell density

Downwelling light intensities at increasing cell densities of *T. suecica* (0, 0.12, 0.47, 0.63, 0.84, 1.13, 1.5, 2.0 x 10^6 cells.ml^{-1}), within the depth range of the test chambers used for the feeding experiments, were measured at 1 cm depth intervals in a blue 25-l plastic vessel because the light sensor size prohibited

measurement directly in the 3-l test chambers. Light intensity at the surface was 8.7-10.1 μmol.s^{-1}.m^{-2}, in keeping with experiments 2 and 3. Turbidity of each density of algae was recorded. The relationship between decreasing light intensity and depth may be described by the general exponential decay equation:

$$I = I_0.e^{-Kd}$$

where irradiance is reduced from I_0 to I in depth d (meters), and K is the attenuation coefficient (Clarke and Backus, 1964; Lythgoe, 1988). Sigmaplot® 5.0 (SPSS Inc.) was used to calculate K for each of the algal cell densities examined.

Upwelling light intensity (reflected light) was measured at 1 cm depth intervals *in situ* in the 3-l test chambers, from the surface to 15 cm depth, at three algal cell densities, 0.63, 0.12, and 0 x 10^6 cells.ml^{-1}, and at 5 cm depth intervals for cell densities 1.5, 1.13, 0.84, and 0.47 x 10^6 cells.ml^{-1}.

For comparison, the light environment in a greenwater larval culture was examined by measuring downwelling and upwelling light intensities at 20 cm depth intervals in a 3000-l larval culture tank, in which surface light intensity was 2-19 μmol.s^{-1}.m^{-2}, *T. suecica* density was 0.08 x 10^6 cells.ml^{-1} and turbidity was 5.39 NTU.

4.3.8 Statistics

A linear regression of larval SL with age, standardised to degree-days (°C days) to accommodate differences in culture temperature, was fitted with Sigmaplot® for each cohort to compare their growth, from days 6 to 25 post-hatching, which covered the period of exogenous feeding. One-way ANOVA was performed using JMP (© SAS Institute Inc.) to analyse the effect of turbidity or light intensity treatments on the proportion of larvae feeding and on larval feeding intensity, within each age for each experiment. Prior to analysis, feeding intensities were standardised to rotifers consumed per feeding larva per minute.

In experiment 1, a planned comparison was made between the 10 $\mu mol.s^{-1}.m^{-2}$ light intensity treatment in clearwater and the greenwater control. Tukey-Kramer multiple comparison of means (P = 0.05) was used when treatment effects were significant. Where necessary, data were transformed by arcsin$\sqrt{}$ for proportion of larvae feeding and $\log_{10}$ for feeding intensity, to satisfy Cochran's test for homogeneity of variance and the Shapiro-Wilk test for normal distribution of residuals (replicate means - treatment means).

4.4 Results

4.4.1 Larval growth

The growth of larvae was similar in the three cohorts irrespective of whether they were reared in clearwater or greenwater conditions; 0.013, 0.011, and 0.012 mm.°C day^{-1}, for cohorts 1, 2 and 3, respectively (Fig.4.1). Larvae hatched at 3.68 ± 0.02 mm SL (n=5) in cohort 1, and the yolksac was absorbed prior to first-feeding on day 7 post-hatching (5.5 mm SL) in all cohorts.

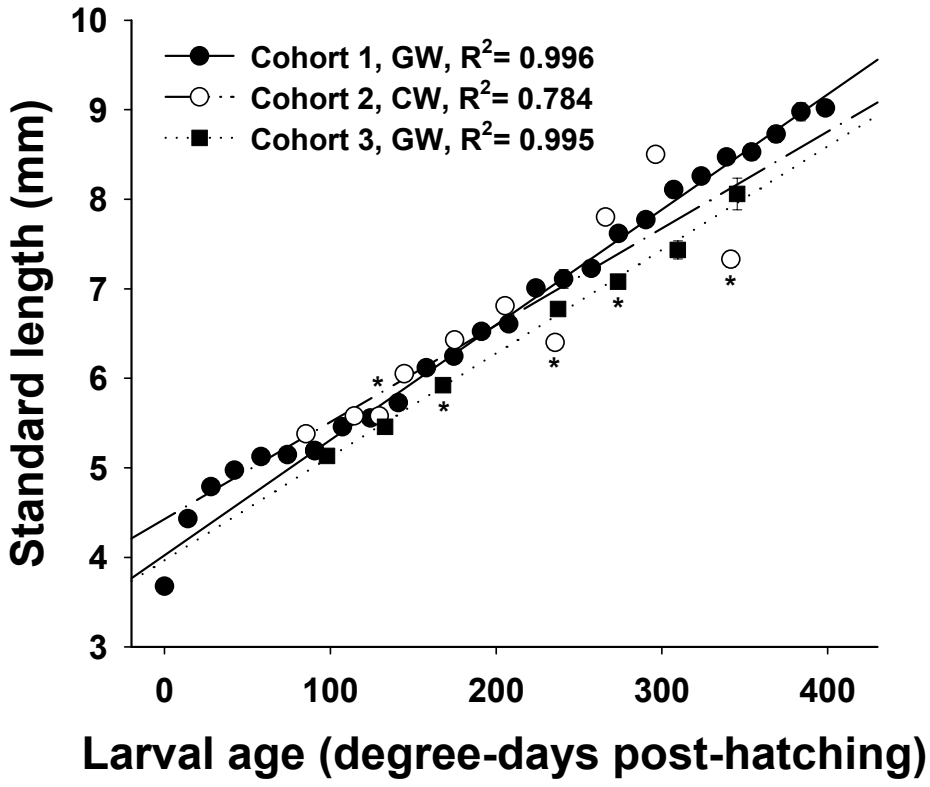

Figure 4.1. Growth of larval striped trumpeter *Latris lineata* with age in degree-days in three cohorts used to assess the effects of light intensity and turbidity on feeding performance. Cohort 1 n = 10, cohorts 2 and 3 n = 5 except where * indicates n = 20 on experiment days. Values are means ± SE. GW - greenwater culture, CW - clearwater culture.

4.4.2 Experiment 1 - The effect of light intensity under clearwater conditions on feeding performance of larvae reared in greenwater

There was a general trend for a higher proportion of striped trumpeter larvae to feed and for feeding intensity to increase with age from day 8 to day 23 post-hatching (Fig.4.2). On day 8 post-hatching, the proportion of larvae feeding and feeding intensity were significantly lower in the lowest light intensity treatment (0.1 $\mu mol.s^{-1}.m^{-2}$) than in the other light intensities tested (ANOVA, N 4 treatments, 5 replicates, $F = 35.62$, df 3, 16, $P < 0.0001$, and N 4, 5, $F = 12.36$, df 3, 16, $P = 0.0002$, respectively). On day 15 post-hatching, the proportion of larvae feeding tended to be lower than on days 8 and 23, however light intensity significantly affected the proportion of larvae feeding (ANOVA, N 4, 5, $F = 4.06$, df 3, 16, $P = 0.025$) in a similar manner to day 8, with an increased proportion of larvae feeding at higher light intensities. Feeding intensity of 15 day old larvae was similar to that on day 8, increasing significantly with higher light intensities (ANOVA, N 4, 5, $F = 24.93$, df 3, 16, $P < 0.0001$). On day 23 post-hatching, neither the proportion of larvae feeding nor larval feeding intensity were significantly different across the range of light intensities tested.

On all days tested, there were no significant differences in the proportion of larvae feeding in the 10 $\mu mol.s^{-1}.m^{-2}$ treatment in clearwater and in the greenwater (10-GW) treatment (Fig.4.2a). However, there was a significantly higher feeding intensity in the 10-GW treatment on day 23 post-hatching compared with the clearwater treatment with equivalent surface light intensity (0.23 ± 0.02 and 0.16 ± 0.01 rotifers consumed.$larva^{-1}.min^{-1}$, respectively) (ANOVA, N 2, 5, $F = 6.20$, df 1, 8, $P = 0.038$), and a trend toward higher feeding intensity in greenwater on days 8 and 15 post-hatching (ANOVA, N 2, 5, $F = 3.52$, df 1, 8, $P = 0.098$, and N 2, 5, $F = 4.19$, df 1, 8, $P = 0.075$, respectively) (Fig.4.2b).

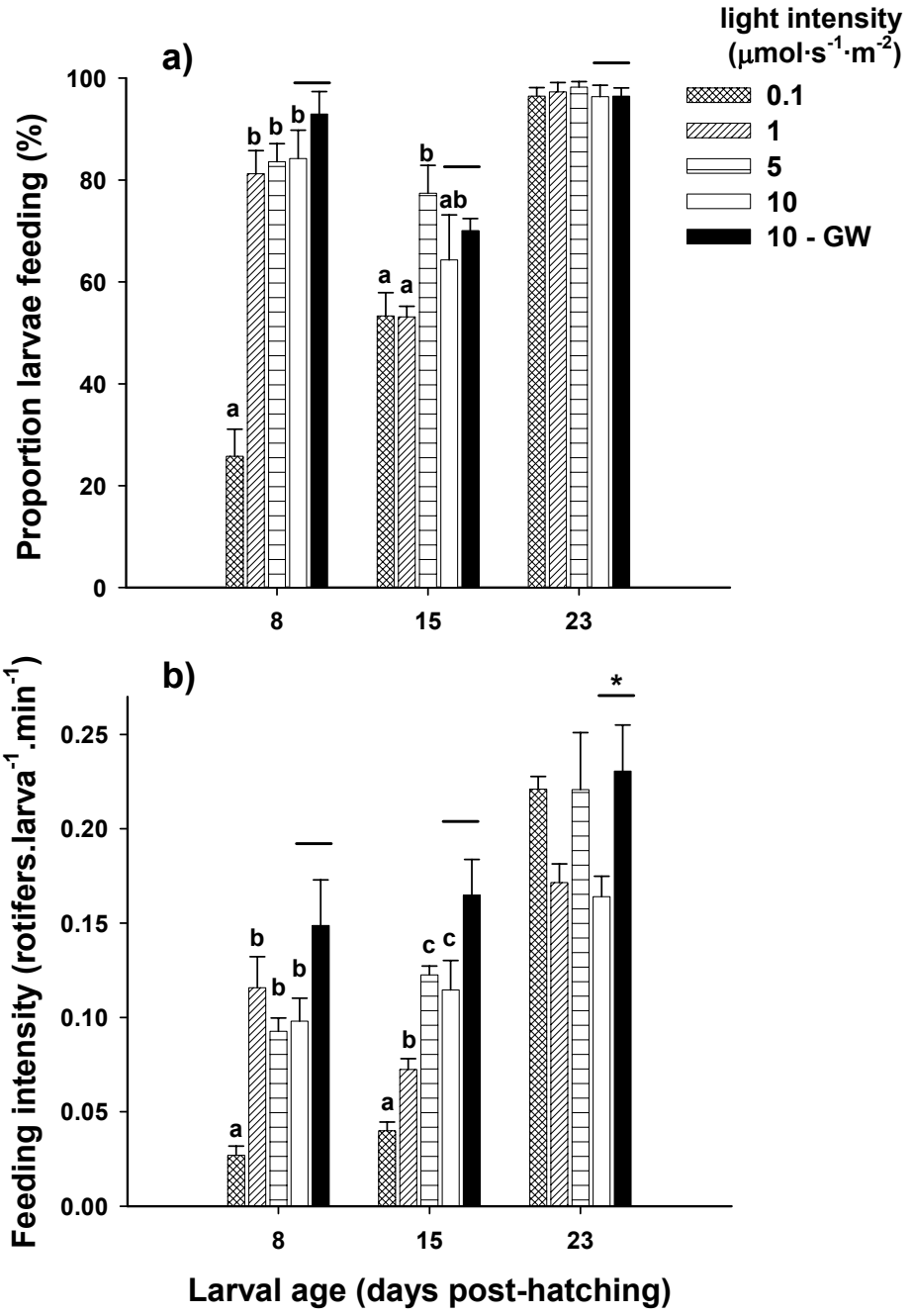

Figure 4.2. Feeding response of striped trumpeter *Latris lineata* larvae in clearwater at four light intensities and in greenwater with a surface light intensity of 10 μmol.s^{-1}.m^{-2} and turbidity of 5.08 ± 0.31 NTU (nephelometric turbidity units) (10-GW), with increasing larval age, a) proportion of larvae feeding and b) feeding intensity. Experiment feeding duration was 1 hour. Means ± SE (n = 5 replicates, 20 larvae per replicate). Different superscripts (a, b, c) within days indicate significant differences among light intensity treatments ($P < 0.05$). Horizontal bars indicate planned daily comparisons between 10 μmol.s^{-1}.m^{-2} clearwater and 10-GW, where * indicates a significant difference between means ($P < 0.05$).

4.4.3 Experiment 2 - The effect of algal cell-induced turbidity on the feeding performance of larvae reared in clearwater

On day 9 post-hatching, the proportion of clearwater-reared larvae feeding at the highest algal cell density was significantly lower than at the other cell density treatments tested (ANOVA, N 4, 5, F = 8.37, df 3, 16, P = 0.0014) (Fig.4.3a). In addition, a stepwise decline in feeding intensity with increasing cell density occurred on day 9, and feeding intensity was significantly higher in clearwater than in the two highest cell density treatments (ANOVA, N 4, 5, F = 11.05, df 3, 16, P = 0.0004) (Fig.4.3b). However, no significant differences in the proportion of larvae feeding or feeding intensity were observed on days 16 and 23 post-hatching in the algae-induced turbidity treatments tested (Fig.4.3).

4.4.4 Experiment 3 - The effect of algal cell-induced turbidity on the feeding performance of larvae reared in greenwater

A higher proportion of larvae reared in greenwater fed in the two higher turbidity treatments than in the clearwater and low cell density treatments on days 10 and 15 post-hatching (ANOVA, N 4, 5, F = 5.44, df 3, 16, P = 0.009, and N 4, 5, F = 5.44, df 3, 16, P < 0.0001, respectively) (Fig.4.4a). Feeding intensity was variable on day 10 post-hatching and there were no significant differences between treatments. However, on day 15 post-hatching a significant difference in feeding intensity was caused by a 3 to 5-fold reduction in intensity between the clearwater and low turbidity treatments compared with the two high turbidity treatments (ANOVA, N 4, 5, F = 48.82, df 3, 16, P < 0.0001) (Fig.4.4b).

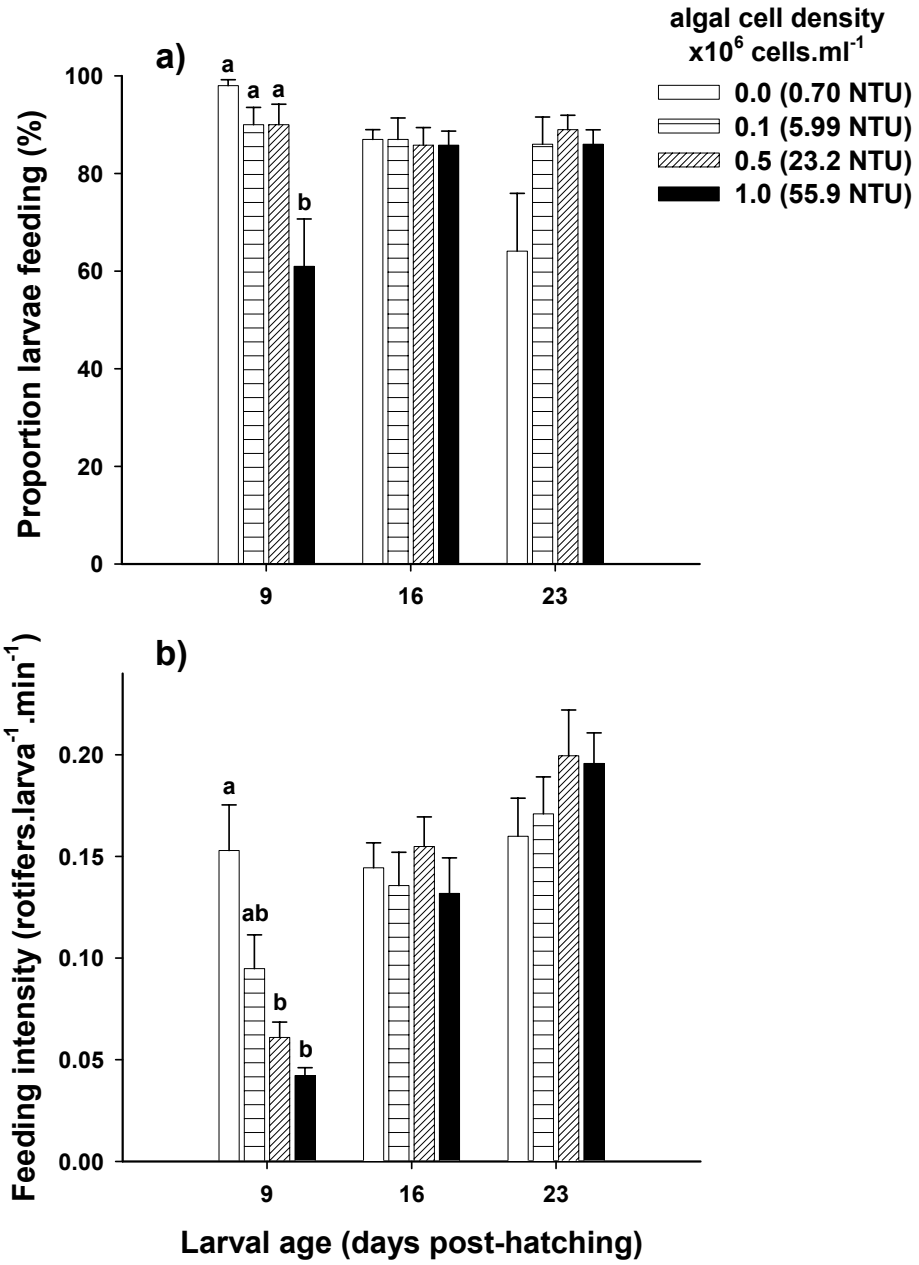

Figure 4.3. Feeding response of striped trumpeter *Latris lineata* larvae reared in clearwater in a range of algal cell densities (turbidities shown in brackets as NTU - nephelometric turbidity units), with increasing larval age, a) proportion of larvae feeding and b) feeding intensity. Experiment feeding duration was 2 hours on days 8 and 15, and 30 minutes on day 23. Means ± SE (n = 5 replicates, 20 larvae per replicate). Different superscripts (a, b) within days indicate significant differences among turbidity treatments ($P < 0.05$).

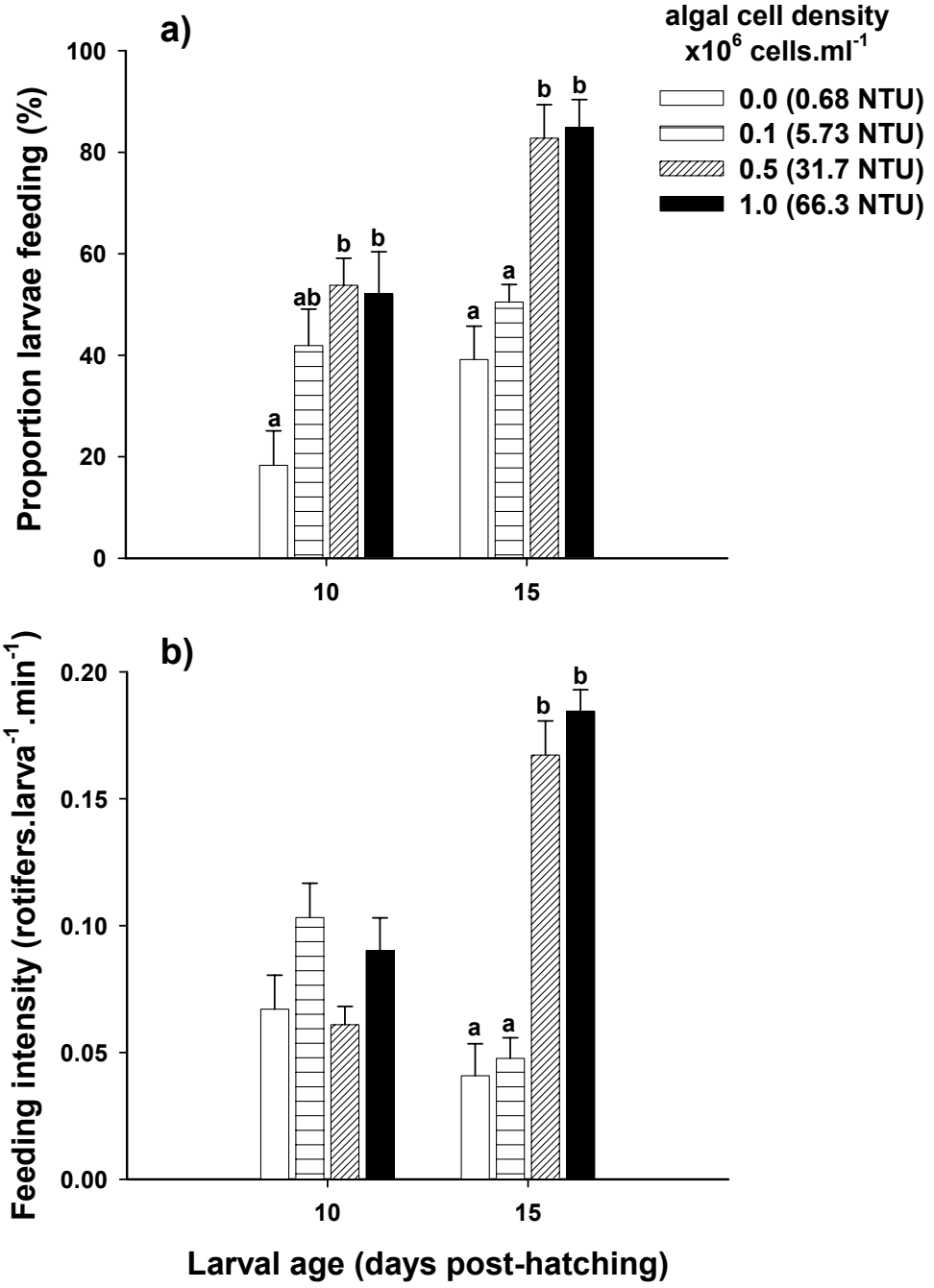

Figure 4.4. Feeding response of striped trumpeter *Latris lineata* larvae reared in greenwater in a range of algal cell densities (turbidities shown in brackets as NTU - nephelometric turbidity units), with increasing larval age, a) proportion of larvae feeding and b) feeding intensity. Experiment feeding duration was 2 hours. Means ± SE (n = 5 replicates, 20 larvae per replicate). Different superscripts (a, b) within days indicate significant differences among means ($P < 0.05$).

4.4.5 Light attenuation and reflected light profiles

Attenuation of light with increasing algal cell density (0 - 2 x 10^6 cells.ml^{-1}) over the depth range of the test chambers was described by exponential decay, with increasing attenuation coefficients (K) of 1.4, 4.7, 10.5, 13.5, 16.4, 24.4, 28.9, and 33.1 for turbidities of 0.2, 4.3, 18.0, 26.5, 35.9, 48.4, 73.1, and 110 NTU, respectively (Fig.4.5a). Light intensity at a depth equivalent to the base of the test chamber (13 cm) ranged between 8.05 μmol.s^{-1}.m^{-2} in clearwater (0.2 NTU) to 0.36 μmol.s^{-1}.m^{-2} at the cell density equivalent to the highest turbidity used in feeding trials (1.5 x 10^6 cells.ml^{-1}, 73.1 NTU). The intensity of reflected, upwelling light was similar at the surface and bottom of the 3-l tanks (15 cm depth) (Fig.4.5b). However, there was a peak of light intensity at a depth of 12 cm, particularly evident in clearwater and low density algae (0.12 x 10^6 cells.ml^{-1}), but was diminished in algae at higher densities. At the scale of the 3-l test chambers, the highest ratio of downwelling to upwelling light at the surface was achieved in the densest algae examined (499.0:1), while the smallest ratio occurred in clearwater (73.2:1).

In a 3000-l larval culture tank, where light intensity was the highest in the centre, light intensity was reduced in an exponential fashion, similar to that seen in the 3-l test chambers, from 16.2 μmol.s^{-1}.m^{-2} just below the surface to 6.8 μmol.s^{-1}.m^{-2} at a depth of 20 cm, declining to less than 1 μmol.s^{-1}.m^{-2} below 80 cm depth. In the darkest corner of the tank, light intensity decreased from 2.6 μmol.s^{-1}.m^{-2} near the surface to 0.4 μmol.s^{-1}.m^{-2} at 60 cm depth. Reflected, upwelling light intensity was low throughout the tank, ranging from 0.08 μmol.s^{-1}.m^{-2} near the surface to 0.02 μmol.s^{-1}.m^{-2} at the bottom of the tank. The highest contrast between downwelling and reflected light intensity was at the surface in the centre of the tank, where the ratio was 202:1. The lowest contrast was a ratio of 20:1 in the darkest corner of the tank at 60 cm depth.

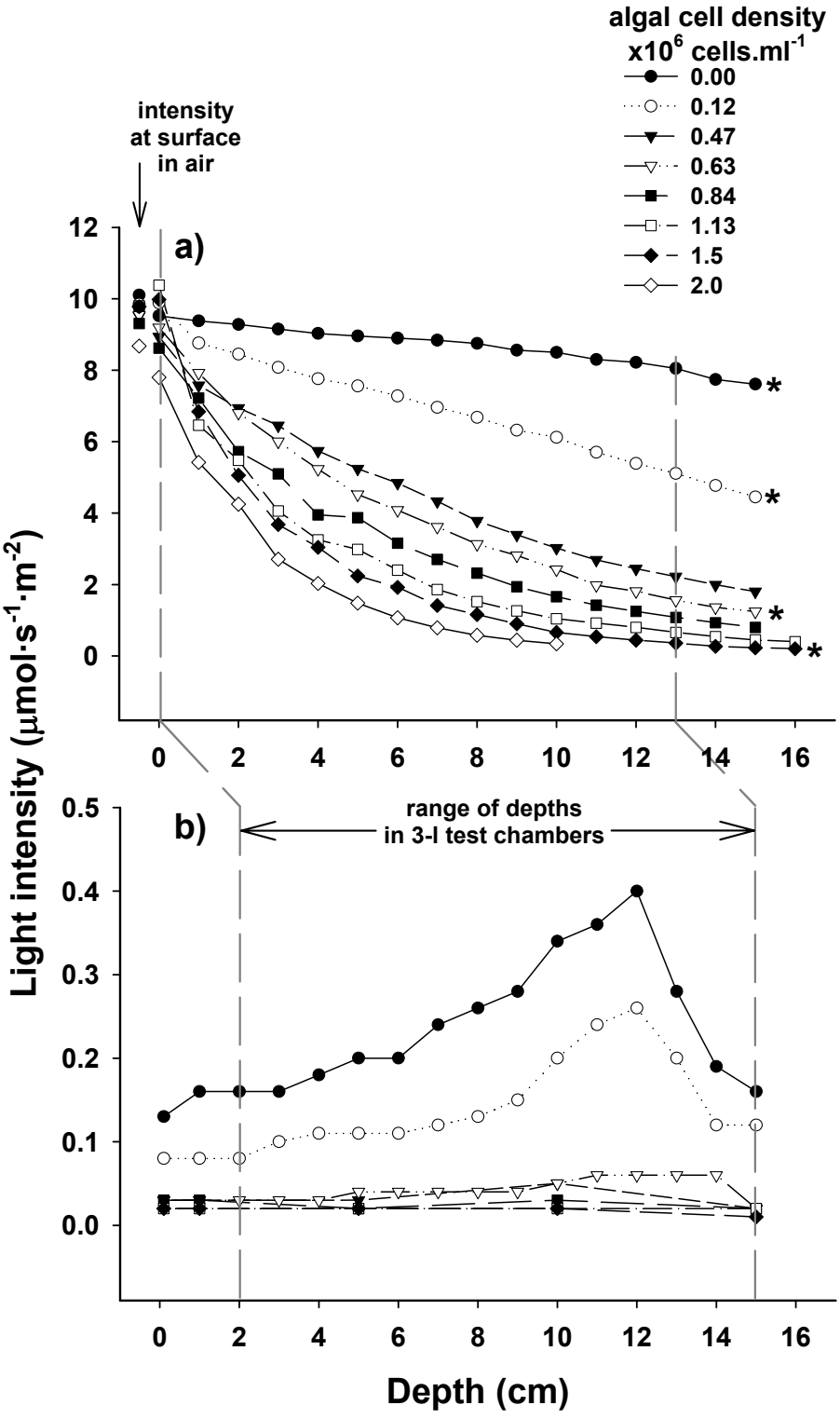

Figure 4.5. Changes in a) downwelling and b) upwelling light intensity with depth in different densities of the alga *Tetraselmis suecica*. * indicate cell densities similar to those used in larval feeding trials.

4.5 Discussion

In this study, striped trumpeter larvae fed at all of the light intensities tested (0.1-10 μmol.s^{-1}.m^{-2}, equivalent to 8-400 lux), indicating this range was above the threshold light intensity level for feeding in this species. The tested range was higher than the average threshold for feeding in young larvae, 0.1 lux, suggested by Blaxter (1986), and higher than the feeding threshold demonstrated for plaice *Pleuronectes platessa* and turbot *Scophthalmus maximus* larvae, less than 0.1 lux (Huse, 1994). In contrast with the youngest larvae examined in the present study, one day after first feeding (day 8 post-hatching), Job and Bellwood (2000) examined seven tropical teleost species and reported a higher first feeding threshold of up to 10 μmol.s^{-1}.m^{-2}. In the latter study, the visual thresholds of the species improved by three to four orders of magnitude by larval settlement to between 0.01 and 0.001 μmol.s^{-1}.m^{-2} (~ 0.7-0.07 lux, using a conversion factor of 14 from Biggs, 1991). This indicated a marked improvement in daytime (photopic) visual sensitivity with age (Job and Bellwood, 2000). An ontogenetic improvement in visual sensitivity has also been shown in other studies of teleost larvae (Blaxter, 1968; Blaxter, 1969). Such an improvement is suggested in striped trumpeter larvae in the present study by the increased feeding capabilities of larvae in the lowest light intensity tested (0.1 μmol.s^{-1}.m^{-2}) between day 8 and day 23 post-hatching (cohort 1). A similar shift, between day 15 and day 28 at 1 lux (~ 0.01 μmol.s^{-1}.m^{-2}, using a conversion factor of 12 for a white fluorescent light source Biggs, 1991), was observed in striped trumpeter larvae by Pankhurst and Hilder (1998). The latter authors suggested that the larger cross-sectional area of double cone photoreceptors identified in the retinae of larvae from day 25 post-hatching, increased the capacity for light capture, thus contributing to increasing photopic visual sensitivity.

Striped trumpeter larvae reared in greenwater (cohort 1, Fig.4.2) generally fed equally well in clearwater at light intensities in the range 1-10 μmol.s^{-1}.m^{-2} (50-400 lux). The only exception occurred on day 15 at 1 μmol.s^{-1}.m^{-2}, when feeding was lower than at the higher light intensities. It is unclear why the proportion of

larvae feeding on day 15 post-hatching was generally less than on day 8 across all light intensity treatments, although those fish that did feed on day 15 displayed a similar feeding intensity to larvae on day 8. A possible explanation for the lower feeding on day 15 is a deficiency in nutrition interfering with larval sensory function and subsequent feeding response. Bell *et al.* (1995) reported nutritional deficiencies of docosahexaenoic acid (DHA) impaired low light (scotopic) visually mediated feeding performance in juvenile herring *C. harengus*, but found no adverse effect on feeding in conditions appropriate for photopic vision. Since the day 15 larvae in this study had a single cone retinae (Pankhurst and Hilder, 1998), responsible for photopic sensitivity, and developing rods that were not yet likely to be functional for scotopic sensitivity (Chapter 2), it is unlikely that their sensitivity in the lower light treatments was affected by a dietary deficiency. There was also no evidence of impaired feeding in older larvae, which fed well at all light intensities. In addition, consistent growth of the cohort did not support a nutritional compromise and as such an undetected intrinsic larval physiological event, or a disturbance in the physical culture environment at, or prior to the time fish were tested, cannot be discounted as factors contributing to reduced feeding performance.

In experiments 2 and 3, feeding performance was generally equivalent in clearwater and algae-induced turbidity treatments, except for young larvae in experiment 2, and older larvae in experiment 3. Clearwater reared larvae (cohort 2, experiment 2, Fig.4.3) fed well in all algae-induced turbidities, except the youngest larvae (day 9 post-hatching), where feeding capabilities (proportion of larvae feeding and feeding intensity) were reduced with increasing turbidity. Light attenuation profiles established for the depth range of the test chambers revealed that the light intensities available to the larvae in the turbidity treatments were within the optimal range for feeding 1-10 $\mu mol.s^{-1}.m^{-2}$ as determined from experiment 1, except for the bottom of the test chamber at the highest turbidity tested, where light levels approached those where feeding was reduced in young larvae. As a result, it is unlikely that the reduced feeding performance of 9 day old larvae (cohort 2, Fig.4.3) was due to algae-induced turbidity reducing the light intensity range below the feeding threshold, rather it

suggests some other mechanism is operating. Since young greenwater reared larvae (day 10 post-hatching, cohort 3, Fig.4.4) fed well in high cell densities, it seems that the previous culture environment influenced the feeding response of larvae (cohorts 2 and 3). This is supported by the fact that greenwater reared larvae fed poorly in the clearwater environment to which they were naive (cohort 3, Fig.4.4). Experience with particular prey types is known to affect the feeding performance of fish larvae, such that feeding is poorer or delayed with novel prey, indicating a learned component to larval feeding behaviour (Salgado and Hoyt, 1996; Cox and Pankhurst, 2000). Most studies of the effect of turbidity on feeding behaviour of fish have employed relatively short acclimation times without prey, such that the fish have no experience of feeding in the novel environment (e.g. 2h, Benfield and Minello, 1996; overnight, Breitburg, 1988; 24h, Gardner, 1981). Reid *et al.* (1999) found no effect of a novel turbid or clear environment on feeding in adult and juvenile largemouth bass *Micropterus salmoides*. In one case where larvae were reared in greenwater, no reference was made to the turbidity of that environment, nor its relationship to the feeding response of larvae to sediment induced turbidity (Boehlert and Morgan, 1985). This study suggests that the previous visual environment of the predator may affect the subsequent feeding response of larvae, however this remains to be tested in concurrent trials with naïve and experienced larvae.

Interestingly, larvae reared in greenwater and tested at a range of light intensities (cohort 1, Fig.4.2) fed equally well in the novel (clearwater) and familiar (greenwater) backgrounds as seen in the planned comparison between clearwater and greenwater (10-GW) treatments. However, due to experimental constraints, this comparison was conducted at only one light intensity, and there was an indication that feeding intensity was higher in greenwater than in clearwater, although this was only significant in larvae at day 23 post-hatching. Miner and Stein (1993), found detrimental effects on larval feeding response with increasing turbidity in low surface light conditions, but not in high light conditions. The interaction between turbidity and surface light intensity remains to be examined in striped trumpeter.

Due to the confounding influence of prior culture history it was not possible to isolate the effect of clearwater and algae-induced turbidity on the visual feeding behaviour of striped trumpeter larvae. In fact, growth rates of the cohorts cultured in greenwater (cohorts 1 and 3) and clearwater (cohort 2) were similar, suggesting that one environment was not more favourable than another for long-term culture. Bristow *et al.* (1996) found that feeding performance, growth, and survival of larval walleye *Stizostedion vitreum* were better in turbid culture than in coloured water with similar light intensity attenuation. The authors suggested that reduced light intensity is not the reason for improved larval performance in turbid water, rather the result implicates other optical properties of the turbid environment, including light scattering and spectral quality. Boehlert and Morgan (1985) proposed two possible mechanisms for enhancement of prey detection by larvae in turbid conditions. Firstly, that light scattered by particles in the water could provide a bright background and increase prey contrast, and secondly that scattered light could illuminate prey from all directions. Enhanced prey contrast was also proposed by Miner and Stein (1993) to explain increased feeding intensity and altered prey selection with increasing environmental turbidity under high surface light conditions in larval bluegill, *Lepomis macrochirus*. In addition, the presence of particulate matter will change the spectral quality of the visual environment and this has also been implicated in altering prey contrast and feeding success of fish. Utne-Palm (1999) demonstrated that the contrast of prey to the background played a major role in the selection of prey by the goby, *Gobiusculus flavescens*, with red and transparent prey being most visible against short-wavelength (blue-green) background illumination. Prey contrast under these conditions will be affected by the relative intensity and colour of light transmitted by the prey, that of the background light and the spectral absorbance characteristics of larval visual pigments. Britt *et al.* (2001) recently identified several different visual pigments occurring in various combinations in 22 species of marine fish larvae. Green-sensitive single cones were the most common, but a high proportion of the species also had ultraviolet-sensitive single cones. Helvik *et al.* (2001) also observed different visual pigments to be expressed in different regions of the retina in Atlantic halibut larvae, presumably optimising colour contrast

sensitivity in different regions of the visual field in accordance with differing properties of downwelling and upwelling light. Because visual pigments of fish can be affected by environmental light conditions and the visual pigments of larval fish change during development (Douglas and Hawryshyn, 1990), the colour and intensity of light in the rearing environment may affect the expression of cone visual pigments. How labile the expression of larval cone visual pigments are to altered environmental light conditions under culture situations has not been examined, but this cannot be discounted as a factor affecting prey contrast and therefore larval feeding success in this study. Other authors have proposed algae may release chemicals stimulating feeding behaviour of larvae in greenwater (Lazo *et al.*, 2000), yet inert particle-induced turbidity where chemicals from algae would not be present has also enhanced feeding (Bristow and Summerfelt, 1994), supporting the theory that the light environment rather than chemical cues must play a major role in improved feeding.

In contrast with studies of larval feeding behaviour, several studies with adult and juvenile fish have reported decreased feeding performance under turbid conditions (e.g. Gardner, 1981; Barrett *et al.*, 1992; Benfield and Minello, 1996; Utne, 1997), although Vandenbyllaardt *et al.* (1991) demonstrated enhanced feeding in juvenile walleye *S. vitreum* with increasing turbidity. Boehlert and Morgan (1985) and Chesney (1989) suggested that the scattering of light in turbid conditions is more likely to degrade the images of prey perceived by larger fish over longer reactive distances than for larvae where reactive distances tend to be small (Wanzenböck and Schiemer, 1989; Browman and O'Brien, 1992). Gregory and Northcote (1993) reported high foraging rates of juvenile chinook salmon, *Oncorhynchus tshawytscha,* at intermediate turbidities and proposed the fish may use turbid water as a refuge from predators while foraging, a theory supported by the predator avoidance behaviour of the juveniles (Gregory, 1993).

The algal cell densities used in the turbidity experiments in this study had high attenuation coefficients (K = 5 to 29) compared with those reported for near

surface (0-100 m) oceanic waters (K = 0.071 to 0.085) (Clarke and Backus, 1964), where striped trumpeter larvae may be found in the wild (Furlani and Ruwald, 1999). In culture, 'unnatural' modifications to environmental factors, such as algal cell density or light intensity, are employed to mask problems with artificial holding systems, for example small tanks with reflective walls (Huse, 1993; Naas *et al.*, 1996; Boeuf and Le Bail, 1999). Naas *et al* (1996) suggested the ratio between downwelling and upwelling light in greenwater tanks (ratio up to 101:1) would be beneficial to the distribution of larvae because it simulates the light conditions in the ocean where the predominant light is downwelling, with almost nothing upwelling (Lythgoe, 1988). In this study, the densest algae used in turbidity trials had a ratio of 500:1 downwelling:upwelling at the surface of the test chambers, and in the culture tank the maximum ratio was 202:1, both ratios appropriately mimic conditions in the wild for larval culture according to Naas *et al.* (1996). Observation of striped trumpeter larvae suggest they are more evenly distributed in greenwater compared with clearwater culture, with less tendency to be closely associated with the walls of the tanks, known as 'wall-nosing' or clinging behaviour, though this behaviour remains to be quantified. Similar distribution patterns have been observed in other studies and have been attributed to different light regimes in clear and turbid conditions (Bristow and Summerfelt, 1994; Rieger and Summerfelt, 1997). Rieger and Summerfelt (1997) suggested that larval clinging behaviour has detrimental affects on feeding and growth. In the small hemispherical test chambers used for feeding trials with striped trumpeter larvae, reflected light from the tank walls and floor was focussed in the centre of the chamber near the bottom in clearwater and low algal cell density. Larval feeding was most evident in the top 2-4 cm in the test chambers (Cobcroft, unpubl. observ.), away from the focus of reflected light. However, this focus of reflected light may have altered larval distribution and feeding, with differential effects dependent upon larval experience of a particular range of downwelling to upwelling irradiance. Thus, another possible mode of action of turbidity on larval feeding response was through the diminishing of the focus of light intensity in the bottom of the test chambers.

A range of feeding durations were used in this study, reflecting increasing larval feeding competency with age. However, feeding durations within days for each experiment were consistent, enabling valid comparison of the feeding responses to different treatments within day. In experiment 2, where a 30 minute feeding duration was used with 23 day old larvae, feeding intensity may be overestimated in relation to the younger larvae allowed 2 hours to feed. This may occur if feeding intensity declines with time after the onset of feeding, as larvae become satiated following starvation. In addition, handling time has been implicated as interfering with treatment effects in feeding trials, particularly in older animals (Gregory and Northcote, 1993). Striped trumpeter larvae display increased feeding with increasing prey density from 2 to 10 rotifers per ml (Cobcroft unpubl. data). Therefore, at the prey density offered in this study, 2 rotifers per ml, it is not likely that handling time would limit the feeding abilities observed.

The present study demonstrates that striped trumpeter larvae feed well in a wide range of light intensities in clearwater. A shift in photopic visual sensitivity occurred with larval age, however further studies with lower light intensities are needed to determine the absolute light intensity threshold for feeding. The results showed that greenwater can provide a visual advantage to feeding in striped trumpeter larvae, although the results were confounded by prior culture environment history. Overall, the effect of algae-induced turbidity on feeding performance was equivalent to that of clearwater. It remains to be demonstrated in concurrent trials if the effect of turbidity on larval feeding behaviour is consistent irrespective of whether it is a novel or a previously experienced feeding environment.

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5. Chapter Five. Jaw development and malformation in cultured striped trumpeter *Latris lineata*

5.1 Abstract

Intensive culture of striped trumpeter (*Latris lineata*) has resulted in a high incidence of jaw malformation in juveniles. In this study, cranial and jaw development in striped trumpeter was described in cultured larvae reared in greenwater on rotifers and *Artemia*. Jaw malformation was only evident in post-flexion larvae greater than 10 mm standard length and was characterised by an open jaw in which the maxilla and premaxilla were aligned dorso-ventrally, and the anterior hyoid arch elements were in an abnormal ventral position. X-radiography of the heads of juvenile and adult cultured striped trumpeter revealed ventro-lateral distortion of the jaw elements in comparison with wild-caught fish. The possible role of physical environmental factors and nutrition during culture are discussed in relation to jaw malformation in this and other species.

Keywords: Striped trumpeter, marine fish larvae, jaw malformation, cranial skeleton

5.2 Introduction

Skeletal malformations can be a problem in cultured teleost species and may cause reduced survival (Barahona-Fernandes, 1982; Andrades *et al.*, 1996) or render product unsaleable (Howell *et al.*, 1998), yet the causes remain poorly understood. In juvenile and adult fish, skeletal malformations have been associated with non-inflation of the swimbladder (Kitajima *et al.*, 1981; Chatain, 1994), nutritional deficiencies (reviews by Dabrowski, 1986; Roberts, 1989; Bruno and Poppe, 1996), disease (Bucke and Andrews, 1985), genetic effects (Sindermann, 1990), and environmental factors such as mechanical stress

(Chatain, 1994). Some skeletal malformations are thought to originate during the labile egg and larval phase, becoming progressively evident in older individuals (Norcross *et al.*, 1996). Development of such skeletal abnormalities has been investigated from the larval stage through to juveniles and adults in some species (Barahona-Fernandes, 1982; Andrades, *et al.*, 1996; Koumoundouros *et al.*, 1997). Deformations in teleost larvae are often reported, being attributed to inappropriate light intensity and spectra (Battaglene *et al.*, 1990; Liu *et al.*, 1994), combinations of inappropriate temperature and/or salinity (Santerre, 1976; Santerre and May, 1977; Ottesen and Bolla, 1998; Bermudes and Ritar, 1999), disease (Morrison and MacDonald, 1995), and pollutants (Rosenthal and Alderdice, 1976; Hose *et al.*, 1996). Exposure to non-ionised ammonia during larviculture is also known to damage cartilage (Guillén *et al.*, 1993), with impacts on subsequent skeletal development likely.

It has been suggested that malformations of the mouth, manifesting in young larvae, have a lethal character (Barahona-Fernandes, 1982) probably linked with starvation of fish due to impaired feeding ability (Pittman *et al.*, 1989). Several forms of mouth malformation have been reported in fish, including crossbite, pugheadedness, sucker mouthed, elongation of the lower jaw (Hickey *et al.*, 1977; Barahona-Fernandes, 1982), double mouth (Swan, 1968), open-jaw (Crouch *et al.*, 1973), lower jaw deformity syndrome (Bruno, 1990; Hughes, 1992; Sadler *et al.*, 2001), and gaping in larvae (McFarlane, 1989; Pittman *et al.*, 1990b; Morrison and MacDonald, 1995).

Striped trumpeter (*Latris lineata*: Latrididae) is currently under investigation at the Tasmanian Aquaculture and Fisheries Institute (TAFI), Marine Research Laboratories (MRL), as a candidate for temperate marine aquaculture in Australia and progress has been achieved in controlling reproduction (Goodsell *et al.*, 1996; Morehead, 1998; Morehead *et al.*, 1998; Pankhurst and Hilder, 1998; Bermudes and Ritar, 1999; Furlani and Ruwald, 1999; Morehead *et al.*, 2000). However, a high proportion of the juveniles cultured to date, exhibit malformations of the swimbladder and vertebrae (Trotter *et al.*, 2001), as well as the opercula and jaw. Juveniles have been successfully reared from five cohorts

to date, with mortality during the larval phase, prior to the appearance of jaw malformation, producing a bottleneck in production (Pankhurst and Hilder, 1998). As jaw malformation may affect the success of culturing striped trumpeter, various factors will need to be tested to determine their involvement in inducing this deformity. An understanding of normal jaw development and the characterisation of abnormal development is required as a first step in solving the problem (Koumoundouros, *et al.*, 1997).

In this study, development of the cranium, jaw and jaw suspensorium in cultured striped trumpeter larvae was described. In addition, jaw malformation was characterised in hatchery-reared post-flexion larvae, juvenile, and adult striped trumpeter, and compared with morphology of the jaw in wild-caught adults.

5.3 Materials and Methods

Striped trumpeter larvae and post-flexion larvae cultured in 1998 and 1997, cohorts 1 and 2 respectively, juveniles from larvae cultured in 1994 and 1997, and wild-caught juveniles on-grown in tanks at the TAFI, MRL were sampled to examine jaw development and malformation.

5.3.1 Larval culture - jaw development

Larvae were sampled from a single batch of eggs (cohort 1) to describe ontogeny of the cranium and jaw. Gametes were collected from 4 male and 2 female broodstock and were fertilised as described by Morehead *et al.* (2001). Egg and yolk-sac incubation occurred under 12-h light/12-h dark photoperiod and otherwise followed that described by Pankhurst and Hilder (1998). Larvae were reared in a 1000-l tank in semi-'greenwater' culture conditions with the addition of the algae *Tetraselmis suecica* and Tahitian *Isochrysis* sp. from 6-14 days post-hatching, and recirculating clearwater thereafter. Salinity was 35 ppt throughout larval culture and temperature was sequentially increased from 14 to 16°C. Standard larval culture hygiene practices were employed. Larvae were fed twice daily with rotifers *Brachionus plicatilis* ($5.ml^{-1}$) and *Artemia* ($1.ml^{-1}$)

enriched with DHA Selco® as per product guidelines. To determine growth, the standard lengths (SL, from tip of snout to end of notochord) of ten larvae (not fixed) were measured on days 5, 8, 10, 12, 14, 16, 18, 20, and 25 post-hatching, and five larvae were measured on day 30.

5.3.2 Bone and cartilage staining

Between three and seven larvae were sampled and fixed in 10% neutral buffered formalin on each of days 17, 22, 30 and 44 post-hatching, while the last ten post-flexion larvae surviving were fixed on day 48 (cohort 1). The total length (TL, from tip of snout to end of caudal fin) of fixed larvae was measured before the larvae were cleared and stained with alcian blue for cartilage and alizarin red S for bone (Taylor and Van Dyke, 1985, in which alcian blue staining was achieved with glacial acetic acid and was proceeded by neutralisation in 1% KOH). Cartilage and bone of the cranium, jaw, and hyoid arch were drawn using a combination of *camera lucida* and tracing of images captured with Scion ImagePC Beta 3b software from a Wild M5 stereo microscope. Bone terminology largely followed Matsuoka (1987), with additions from Bond (1996) and Koumoundouros *et al.* (2000).

5.3.3 Jaw malformation in post-flexion larvae

Two post-flexion larvae (day 62 post-hatching), representative of malformed larvae in a second cohort (cohort 2), were sampled from a 1000-l semi-'greenwater' culture in July, 1997. Larvae were fixed in 10% neutral buffered formalin, then cleared and stained as described above.

5.3.4 Radiography

The skeletal structures of the jaws in cultured and wild-caught fish were compared using x-radiography. Juveniles sampled from cohort 2 (1 year 9 months of age, n = 12), and from a cohort cultured in 1994 (4 years 5 months of

age, n = 9), and adults wild-caught as juveniles in 1997 and ongrown at the MRL (n = 6), were examined. The fish were anaesthetised in a solution of 0.02% 2-phenoxyethanol in seawater before x-radiographs of their heads were taken (Atomscope 100P, settings 60 kVp, 30 mA, and 0.04-0.08 s exposure time). In addition, the lower jaws of malformed fish (approximately 3 years of age from a cohort cultured in 1994, n = 4) preserved in 10% neutral buffered formalin were dissected, and ventral and frontal x-radiographs were taken.

5.4 Results

5.4.1 Larval growth

Striped trumpeter larvae from cohort 1 hatched 6 days post-fertilisation at 3.62 ± 0.02 mm SL. Eye pigmentation had commenced by day 5 and was complete by first-feeding on day 7 post-hatching, when the mouth was also functional and the yolk sac had been absorbed (Fig.5.1). The oil drop was absorbed prior to swimbladder inflation, which was evident from day 10 post-hatching. The incidence of swimbladder inflation by day 16 post-hatching was 90% (n = 10). The commencement of flexion, indicated by the dorsal turning of the notochord tip and fin ray development, was observed in larvae over 8 mm SL from day 25 post-hatching. Growth, indicated by the rate of increase in SL of larvae, from day 5 to day 30 post-hatching was 0.132 $mm.day^{-1}$, larvae reaching an average SL of 8.23 ± 0.37 mm (n = 5) by day 30 post-hatching (cohort 1, Fig.5.1). Larvae and post-flexion larvae were observed swimming vigorously around and against the walls of the culture tank (‘wall-nosing’), particularly after the greenwater phase, from day 15 post-hatching. Malformation of the jaw was not evident in live larvae until they reached 10 mm SL.

5.4.2 Skeletal morphology of larval cranium and jaw

Most of the structures of the neurocranium (Fig.5.2) are bilaterally symmetrical and as such only a lateral view of the right-hand side of the head is drawn, except where specified otherwise.

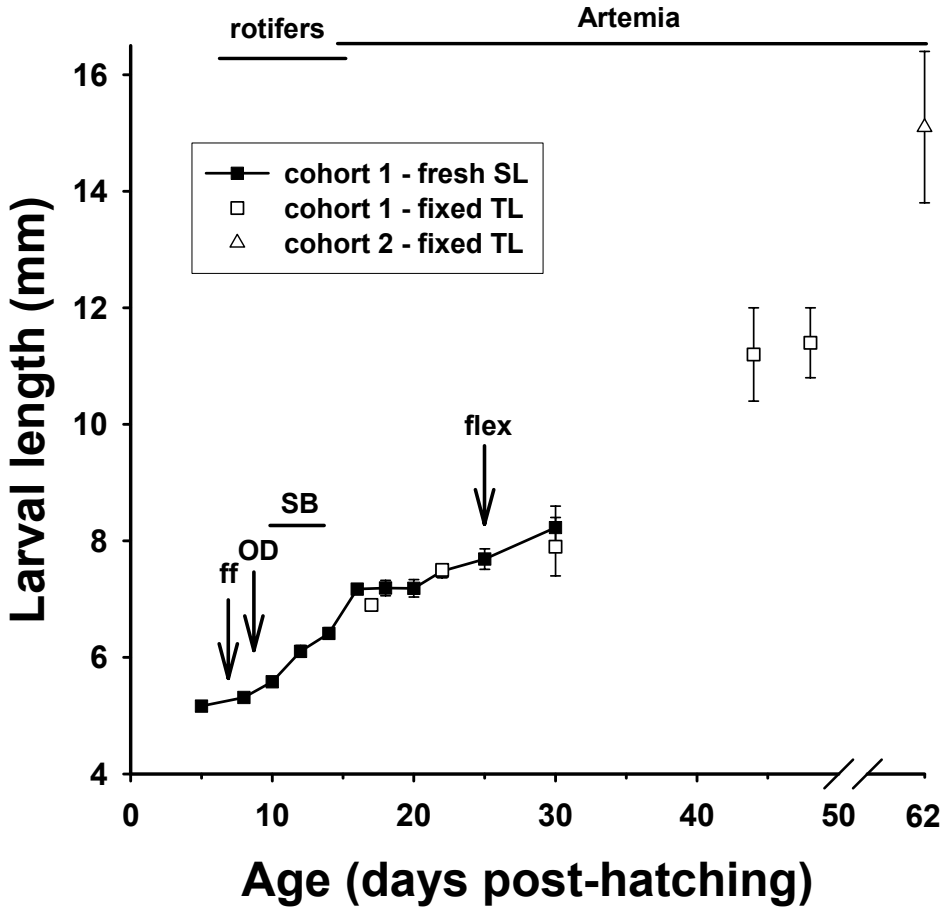


Figure 5.1. Increase in length of striped trumpeter (*Latris lineata*) larvae cultured to examine cranium and jaw development (mean ± SE). Closed squares, standard length (SL) live larvae from cohort 1, n = 10, except day 30 post-hatching where n = 5. Open squares, total length (TL) neutral buffered formalin fixed larvae from cohort 1, n = 3-7. Open triangle, TL neutral buffered formalin fixed larvae from cohort 2, n = 2. Abbreviations: Artemia, period of *Artemia* addition; ff, first-feeding; flex, start of flexion; OD, end of oil drop absorption; rotifers, period of rotifer addition; SB, swimbladder inflation.

5.4.2.1 Cranium

By day 17 post-hatching the cartilaginous sclerotic was present (drawn in Fig.5.2a only for simplicity). The anterior tips of the trabecula were fused to form the cartilaginous ethmoid plate and the posterior lateral edge of the parachordal cartilage extended to the developing cartilaginous auditory capsule (Fig.5.2a). The cartilaginous precursors of the basioccipital and exoccipital had started to form at this stage. In addition, the cartilaginous epiphysial tectum was present and the supraorbital cartilages and ectethmoid bars had started to develop ventrally from the epiphysial tectum, in posterior and anterior directions, respectively.

In day 22 larvae (Fig.5.2b) the supraorbital extended posteriorly to the auditory capsules which were further expanded.

Posterior processes of the ethmoid plate, forming the ventral end of the ectethmoid bars, were apparent in larvae at day 30 post-hatching (Fig.5.2c). In addition, the frontal bones were now visible posterior to the epiphysial tectum.

In day 44 larvae, cartilage of the auditory canals was now fully delineated (Fig.5.2d-e). The posterior end of the trabecula was reduced and supported by the osseous parasphenoid. The medial tectum (*taenia tecta medialis*) was present along the dorsal median line of the cranium between the auditory capsules and the epiphysial tectum. The ectethmoid bars were fully delineated, extending between the epiphysial tectum and ethmoid cartilage. Olfactory capsules were evident in the ethmoid cartilage. The epiphysial tectum was further delineated and extended anteriorly with signs of ossification.

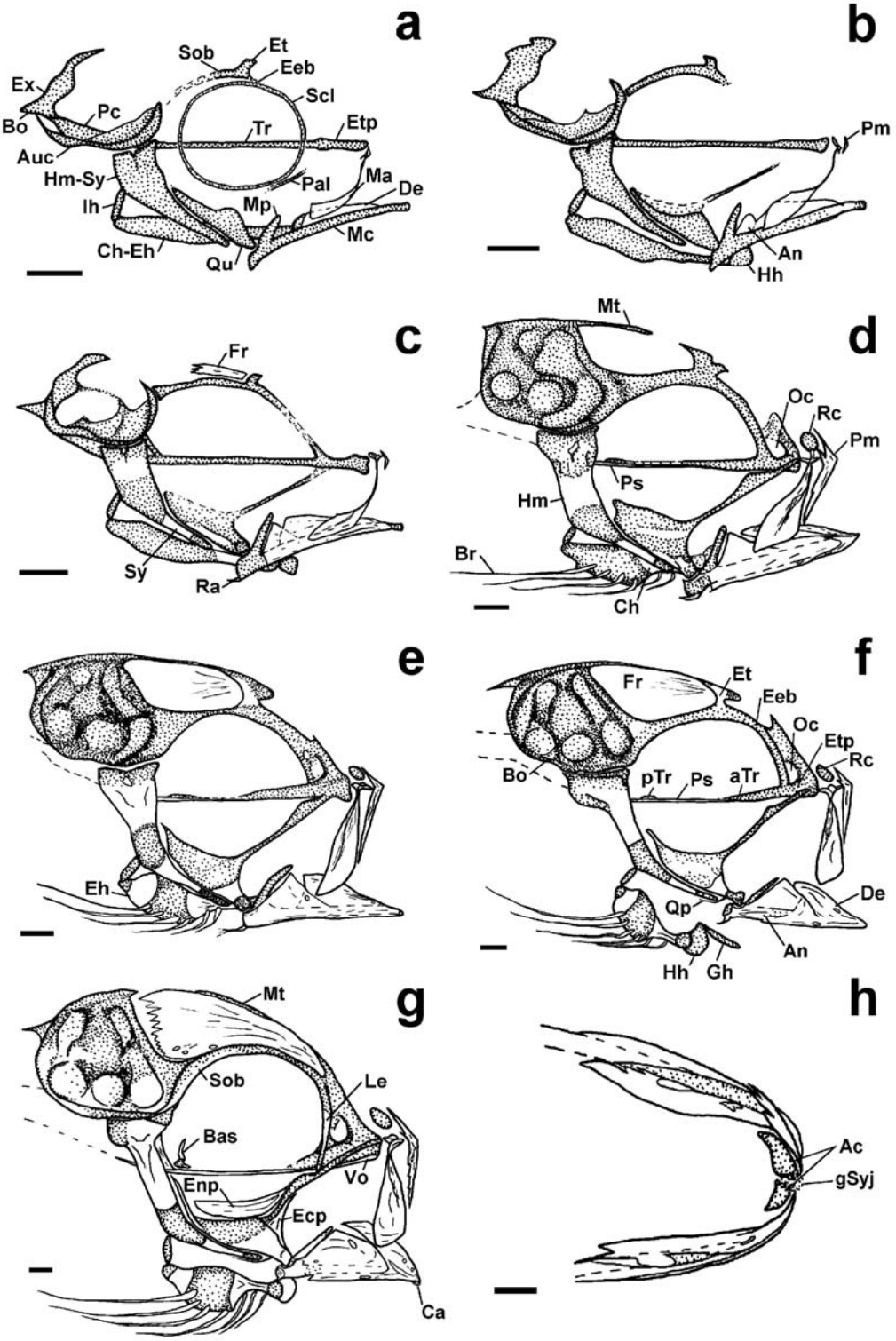
In 48 day old larvae from cohort 1 (Fig.5.2f), all cartilage components of the cranium appeared fully delineated and normal. The parasphenoid had lengthened anteriorly, and remnants of the trabecula existed at the base of the cranium. The ethmoid cartilage and anterior remnants of the trabecula extended posteriorly to one third the length of the parasphenoid. The basioccipital had started to ossify. The ethmoid cartilage was dominated by the olfactory capsules and extended

dorsally to half the length of the ectethmoid bar, towards the epiphysial tectum. The rostral cartilage had enlarged slightly.

In 62 day old post-flexion larvae from cohort 2 (Fig.5.2g), the frontal bones had enlarged anteriorly, covering the epiphysial tectum, and meeting at the dorsal medial line of the cranium, where the posterior of the medial tectum was reduced. Ossification was evident in the lateral ethmoid, supraorbital, and auditory capsule, and the osseous basisphenoid was present at the posterior of the parasphenoid, while the vomer bone was present ventral to the ethmoid plate.

5.4.2.2 Upper jaw, lower jaw and jaw suspensorium

By day 17 post-hatching (Fig.5.2a), the upper jaw was supported by the maxilla, consisting of a thin strip of unossified bone matrix that had a developing articulation surface at the dorsal tip. The Meckel's cartilage, framing the lower jaw, was fully delineated with a dorsal process anterior to the point of articulation with the quadrate. At this stage, the dentary formed a plate parallel to the external lateral surface of the Meckel's cartilage. The hyomandibular-symplectic cartilage extended from the ventro-lateral edge of the auditory capsule to support the posterior edge of the quadrate cartilage. The hypohyal, ceratohyal-epihyal and interhyal cartilages were also present. The palatine had started to develop at this stage, as indicated by the accumulation of dense granular cartilage between but not connecting the ethmoid plate and the quadrate.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In 22 day old larvae (Fig.5.2b), the premaxilla was present, anterior to the dorsal tip of the maxilla. Both the maxilla and premaxilla had started to ossify, indicated by streaks of red stain in the bone elements. The posterior end of the maxilla and the dentary were further developed at this stage, indicated by increased dorso-ventral depth, and ossification of the upper edge of the dentary was evident. The dentary was positioned medially relative to the maxilla when the mouth was closed. The angular bone had developed posterior to the dentary and external to the lateral surface of the Meckel's cartilage. In addition, the palatine had developed posterio-ventrally toward the quadrate.

Ossification of the maxilla and development of the angular bone were more extensive in 30 day old larvae (Fig.5.2c). Tendinous ossification extended back from the posterior ventral tip of the Meckel's cartilage to form the retroarticular bone. The symplectic had differentiated from the hyomandibular by day 30 post-hatching, at which time ossification of both structures had commenced. Also, the quadrate had developed anteriorly and dorsally to form a plate fused anteriorly with the palatine, and the ceratohyal had started to ossify at this stage.

Figure 5.2. *Camera lucida* drawings of the lateral view of cartilages (stippled) and bones (non-stippled) of the cranial skeleton of cleared and stained cultured striped trumpeter larvae, normal development on days 17 (a), 22 (b), 30 (c), and 44 (d), and jaw malformation evident on days 44 (e), 48 (f), and 62 (g) post-hatching, and ventral view of the anterior lower jaw (h) of day 62 larva drawn in (g). Sclerotic is omitted from b-g. Abbreviations: An, angular; Ac, asymmetric cartilage; aTr, anterior remnants of trabecula; Auc, auditory capsule; Bas, basisphenoid; Bo, Basioccipital; Br, branchiostegal ray; Ca, extraneous cartilage ventral to symphysial joint; Ch, ceratohyal; Ch-Eh, ceratohyal-epihyal cartilage; De, dentary; Ecp, ectopterygoid; Eeb, ectethmoid bar; Eh, epihyal; Enp, endopterygoid; Et, epiphysial tectum; Etp, ethmoid plate; Ex, exoccipital; Fr, frontal; Gh, glossohyal; gSyj, gap at symphysial joint; Hh, hypohyal; Hm, hyomandibular; Hm-Sy, hyomandibular-symplectic cartilage; Ih, interhyal; Le, lateral ethmoid; Ma, maxilla; Mc, Meckel's cartilage; Mp, Meckel's dorsal process; Mt, medial tectum (*taenia tecta medialis*); Oc, olfactory capsule; Pal, palatine; Pc, parachordal cartilage; Pm, premaxilla; Ps, parasphenoid; pTr, posterior remnants of trabecula; Qp, quadrate posterior process; Qu, quadrate; Ra, retroarticular; Rc, rostral cartilage; Scl, sclerotic; Sob, supraorbital; Sy, symplectic; Tr, trabecula; Vo, vomer. Scale bars are 200 μm. Larvae drawn are representative of each age, in which mean SL ± SE and sample number (n) were a) 6.9 ± 0.1 mm (n = 7), b) 7.5 ± 0.1 mm (n = 5), c) 7.9 ± 0.5 mm (n = 5), d) 9.3 mm, e) 11.2 ± 0.8 mm (n = 3), f) 11.4 ± 0.6 mm (n = 10), and g) & h) 15.1 ± 1.3 mm (n = 2).

Development of the jaw and jaw suspensorium in striped trumpeter larvae appeared normal to day 30 post-hatching, as indicated by normal jaw articulation observed during feeding in live larvae (8.2 ± 0.4 mm, live SL). Malformation of the jaw, characterised by a permanently open mouth, was evident in most post-flexion larvae greater than 10 mm SL, from day 44 (Fig.5.2e-h). The maxilla and premaxilla were dorso-ventrally aligned and resisted backward movement toward the palatine, when pressure was applied. In some cases, the hyoid arch was angled ventrally, so that the hypohyal and glossohyal were well below the lower jaw (Fig.5.2f). Otherwise, malformed larvae appeared to have 'normal' bone structure in terms of size and shape with no obvious fusing of bones.

In 44 day old post-flexion larvae (Fig.5.2d-e), a spherical rostral cartilage was present anterior and dorsal to the ethmoid plate, acting as an articulation surface for the dorsal tip of the maxilla. Both maxilla and premaxilla had developed posterior processes at the dorsal tip, which extended the articulation surface in contact with the rostral cartilage. The premaxilla was associated with the dorsal surface of the rostral cartilage and extended posterio-ventrally (or ventrally, depending on the angle of the maxilla in malformed larvae) to between half and two-thirds the length of the maxilla. By this stage, the ossified dentary was well-delineated and extended posteriorly from the symphysial joint to approximately two-thirds the length of Meckel's cartilage, and dorsally to the height of the dorsal process of Meckel's cartilage. The angular bone extended anteriorly from the Meckel's cartilage dorsal process toward the dentary, and posteriorly beyond the articulation joint, supporting the Meckel's cartilage ventrally. The anterior tip of the palatine extended past the ethmoid cartilage at this stage. Ossification was evident in the connective tissue binding the symplectic to the quadrate, and in the quadrate near the articulation joint with the Meckel's cartilage. The articulation surface of the quadrate was well-delineated. The ceratohyal had differentiated from the epihyal at this stage, and ossification of the epihyal was apparent. Six osseous branchiostegal rays extended posteriorly from the epihyal and ceratohyal at this stage, and the glossohyal was present.

In 48 day old post-flexion larvae the dorsal process of the Meckel's cartilage extended along the posterior edge of the angular bone (Fig.5.2f). Staining indicated the Meckel's cartilage extended anteriorly to approximately half of the dentary (about 7/8 length of lower jaw), but not to the symphysial joint. Of the ten cleared and stained 48 day old larvae, three had lower jaws broken at the symphysial joint. The retroarticular bone had increased in size and was more strongly ossified by this stage. Both dentary and angular were well-delineated and ossified. The posterior tip of the angular supported the articulation joint between the Meckel's cartilage and the quadrate. A posterior process of the quadrate was present, formed by ossification of connective tissue supporting the symplectic.

In 62 day old post-flexion larvae from cohort 2, the dentary and angular appeared weakly developed (Fig.5.2g), with thin osseous layers, compared to an apparently normal individual with a functional jaw from cohort 1, day 44 (Fig.5.2d). Asymmetrically shaped cartilage tissue had developed outside the dentary, ventrally supporting the symphysial joint (Fig.5.2g-h). Uneven development of the Meckel's cartilage within the anterior tip of the dentary, at the symphysial joint, was also observed. A wide gap was present between dentary bones at the symphysial joint. One individual had a bone fragment within the joint (not shown). The osseous endopterygoid and ectopterygoid were present dorsal and ventral to the palatine, respectively, in 62 day old larvae (Fig.5.2g). In addition, ossification was evident in the quadrate, at the point of articulation with the Meckel's cartilage, and in the hypohyal.

No teeth were observed in any of the cleared and stained specimens examined.

5.4.3 Skeletal morphology of juvenile and adult cranium and jaw

All adult and juvenile cultured striped trumpeter examined by radiography had malformed jaws. The degree of malformation among cultured fish was variable when compared with wild-caught fish (Fig.5.3a). In wild-caught fish, the ceratohyal, hypohyal, and glossohyal were located medially within the frame of the lower jaw, supporting the mouth cavity ventrally. One cultured individual had an enlarged maxilla, but otherwise an apparently functional jaw (Fig.5.3b). In extreme cases of malformation, the lower jaw, including Meckel's cartilage, angular and dentary bones, was bent ventrally, temporally, and laterally, so that the mouth was permanently agape with a narrow dorso-ventral opening (Fig.5.3c). The anterior of the quadrate was also in a more dorsal position than

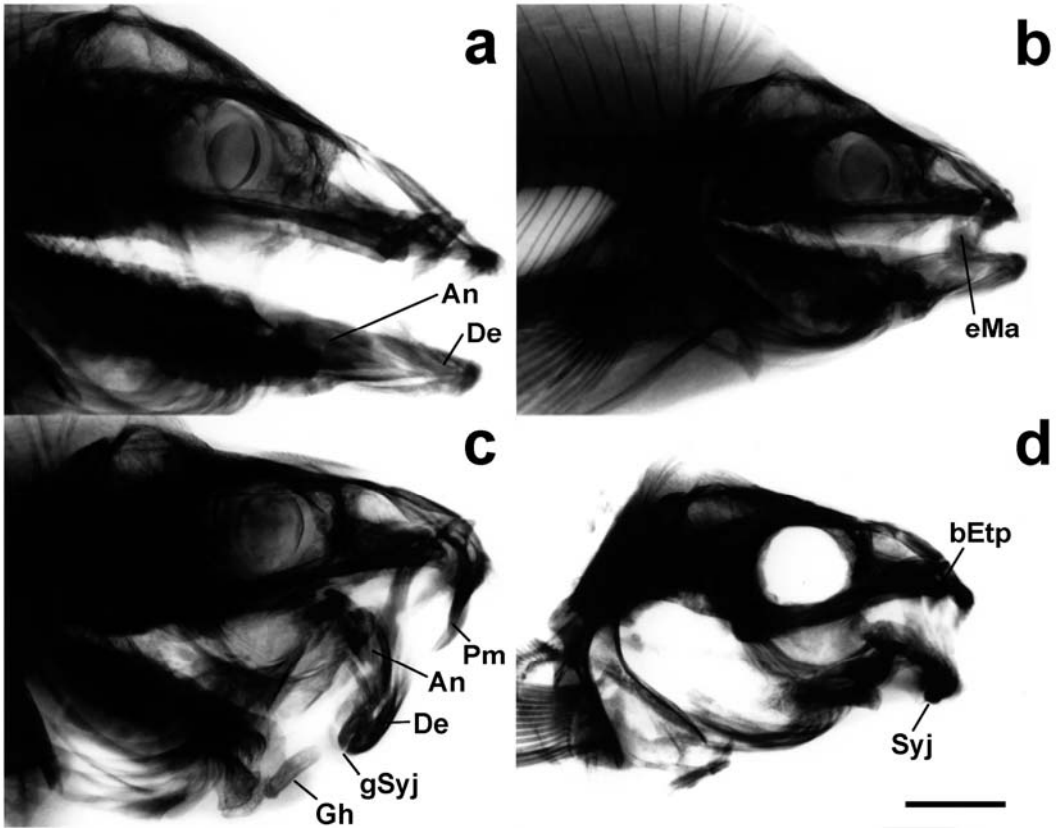

Figure 5.3. X-radiographs of the lateral view of the cranial skeleton of striped trumpeter. a) a normal wild caught adult (47.0 cm FL, 2105 g), b) a relatively normal cultured juvenile (31.0 cm FL, 470 g), c) a malformed cultured juvenile (41.5 cm FL, 950 g), and d) a malformed cultured juvenile (fixed head with eyes removed) (32.0 cm FL, 390 g). Abbreviations: An, angular; bEtp, ethmoid plate bent ventrally; De, dentary; eMa, enlarged maxilla; Gh, glossohyal; gSyj, abnormal gap between adjacent dentary bones at the symphysial joint; Pm, premaxilla; Syj, symphysial joint. Scale bar is 2 cm.

in the wild fish and the ceratohyal was angled ventrally, with the trabecula and parasphenoid angled dorsally from the base of the cranium. The anterior of the ethmoid and vomer bones were bent ventrally in other specimens (Fig.5.3d).

X-radiographs of lower jaws dissected from fixed specimens with malformed jaws revealed asymmetric bones on either side of the symphysial joint (Fig.5.4a-h). Gaps between the dentary bones were apparent (Fig.5.4d,e&g), along with twisting and bending of the dentary and angular bones (Fig.5.4b&f).

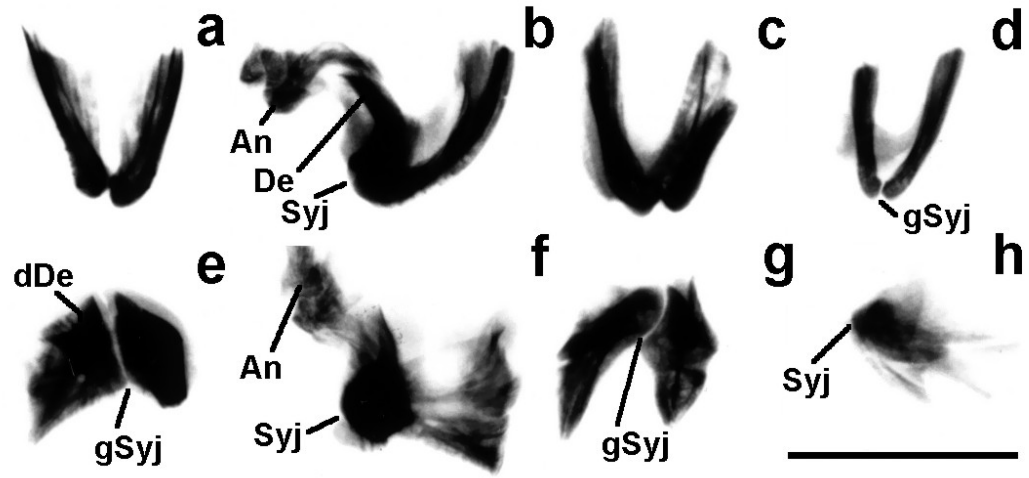

Figure 5.4. X-radiographs of the dorsal (a-d) and frontal (e-h) view of the anterior tip of the lower jaw dissected from four malformed, cultured striped trumpeter juveniles. Abbreviations: An, angular; dDe, dentary with abnormal increase in dorso-ventral depth; De, dentary; gSyj, abnormal gap between adjacent dentary bones at the symphysial joint; Syj, symphysial joint. Scale bar is 2 cm.

5.5 Discussion

Development of the striped trumpeter cranium and jaw was similar to other marine teleost species, including the red sea bream *Pagrus major* (Matsuoka, 1987), sea bass *Lates calcarifer* (Kohno *et al.*, 1996), and sea bream *Archosargus rhomboidalis* (Houde and Potthoff, 1976). However, the development of teeth (not present in any striped trumpeter examined prior to and including day 62 post-hatching), appeared delayed compared with sea bream, red sea bream, sea bass, common dentex (*Dentex dentex*), Atlantic halibut (*Hippoglossus hippoglossus*) and turbot (*Scophthalmus maximus*) in which teeth were present in pre-flexion larvae (Houde and Potthoff, 1976; Matsuoka, 1987; Pittman *et al.*, 1990b; Kohno *et al.*, 1996; Wagemans *et al.*, 1998; Koumoundouros *et al.*, 2000). The late development of teeth, required for biting during prey capture (Kohno *et al.*, 1996), suggests striped trumpeter have a prolonged planktivorous phase, where the mode of visual particulate feeding is by suction, possibly in combination with darting (Lazzaro, 1987; Kohno *et al.*, 1996). This mode of feeding is dependent upon the opening and closing of the mouth (Drost, 1987; Lazzaro, 1987; Kohno *et al.*, 1996) and its effectiveness would be compromised by a fused open jaw in post-flexion larvae. All juveniles cultured to date have exhibited jaw malformation, indicating this deformity is not always lethal, although high attrition during the juvenile phase (Morehead *et al.*, 1999) may be attributed to the progressive inability of malformed fish to feed either on live prey or inert particulate diets. It is unlikely however, that the growth of striped trumpeter larvae was impaired by jaw malformation prior to flexion, as all skeletal elements appeared normal and the mouth was capable of successful feeding at that stage.

Jaw malformation was identified in post-flexion striped trumpeter larvae as a locked open jaw in which the maxilla and premaxilla were aligned dorso-ventrally, rather than posterio-ventrally, and the anterior hyoid arch elements were orientated in an abnormal ventral position. In malformed post-flexion larvae, the symphysial joint appeared compromised due to poor development of the dentary bones at either side, and some cleared and stained specimens had

lower jaws broken at this point. Physical damage caused by wall-nosing of larvae and post-flexion larvae may be responsible for breakages at the symphysial joint, particularly if the joint was already weak, and differential damage to individuals may explain variations in the jaw malformation observed in juveniles. Wall-nosing has been associated with tank colour and light conditions in larval rearing tanks (Bristow and Summerfelt, 1994), and modified lighting conditions, including increased use of greenwater culture, may resolve this problem in striped trumpeter culture. The presence of asymmetric extraneous cartilage around the symphysial joint, which may have been formed in response to skeletal damage, may compensate for the scant support of the dentary bones and the wide gap present in older post-flexion larvae. This asymmetry at the symphysial joint was also present in x-radiographs of cultured juveniles. The extent of jaw malformation in cultured striped trumpeter was however more extreme in the juveniles, with bending and twisting of the jaw and suspensory structures. The mechanism by which this type of malformation occurs may be related to the torsion created by soft tissues (Swan, 1968). If the weakened symphysial joint in young striped trumpeter were damaged, or unable to support the protractor muscles between the lower jaw and the hyoid arch, then the unopposed action of the retractor muscles may pull the hyoid arch into the abnormal ventrally directed position, as reported in double-mouth deformity in trout (*Salmo trutta*) (Swan, 1968). In addition, the connective tissue around the lower jaw may be pulled ventrally by the hyoid arch before the bones are completely developed, to create the bent and twisted lower jaws seen in the malformed striped trumpeter juveniles.

Jaw and other skeletal malformations have been reported in both cultured and wild- caught teleosts (Hickey, 1972; Rosenthal and Alderdice, 1976; Hickey *et al.*, 1977; Barahona-Fernandes, 1982); however, the incidence of deformity may be higher in cultured fish in which selective mortality of deformed fish would be reduced compared to natural populations (Hickey *et al.*, 1977; Barahona-Fernandes, 1982; Koumoundouros *et al.*, 1997; Howell *et al.*, 1998). Several physical parameters have been associated with jaw malformation in cultured larvae, including inappropriate incubation temperature of eggs and yolksac

larvae (Atlantic halibut (Bolla and Holmefjord, 1988; Pittman *et al.*, 1989; Pittman *et al.*, 1990a; Lein *et al.*, 1997; Ottesen and Bolla, 1998); gilthead sea bream *Sparus aurata* (Polo *et al.*, 1991); and sablefish *Anoplopoma fimbria* (McFarlane, 1989)), salinity (Atlantic halibut (Ottesen and Bolla, 1998)), light intensity (Atlantic halibut (Bolla and Holmefjord, 1988)), and bacterial invasion of physically damaged oral membranes (Atlantic halibut (Morrison and MacDonald, 1995)). Further to this, Koumoundouros *et al.* (1997) proposed that intensive rearing conditions and possibly unspecified pollutants may have been involved in modulating gene expression, leading to caudal fin malformation in gilthead sea bream, compared with fish reared extensively in a more 'natural' mesocosm. It is apparent that a systematic multi-factor experimental approach will be needed to determine whether a single culture parameter, or indeed an interaction of physical culture parameters are contributing to the prevalence of jaw deformity in cultured striped trumpeter.

Another possibility is that nutritional factors may be involved in the jaw malformation in striped trumpeter since it manifested after the larvae had been feeding for some time. In terms of highly unsaturated fatty acids (HUFA), striped trumpeter larvae may have a requirement for a higher docosahexaenoic acid (DHA): eicosapentaenoic acid (EPA) ratio than 2:1 which is generally recommended for marine fish larvae (Sargent *et al.*, 1999), since the eggs of wild-caught fish contain 3:1 DHA:EPA (Morehead *et al.*, 2001) and the flesh of wild-caught adults contains 11.6:1 DHA:EPA (Nichols *et al.*, 1994). Although the live feeds provided in this study were boosted with HUFA, these may not have been adequate to meet high nutritional demands at metamorphosis, resulting in jaw and other malformations (Dabrowski, 1986). The incidence of cranial malformations in larvae of other species has been related to insufficient dietary phospholipids (jaw in ayu, *Plecoglossus altivelis* (Kanazawa *et al.*, 1981)), insufficient HUFA and vitamin C (opercular deformities in milkfish, *Chanos chanos* (Gapasin *et al.*, 1998)), while larval skeletal malformations have been associated with an excess of vitamin A and vitamin A metabolites (Japanese flounder, *Paralichthys olivaceus* (Takeuchi *et al.*, 1995; Takeuchi *et al.*, 1998)), and deficiency of vitamin C (lake whitefish, *Coregonus clupeaformis*

(Zitzow and Millard, 1988)). In addition, indirect nutritional deficiencies, through disturbance of digestive physiology or problems with hygiene in the production of diets, have been implicated in scoliosis and lordosis in juvenile sea bass (Person-Le Ruyet, 1989). Current nutritional protocols including provision of HUFA enriched rotifers and *Artemia* (this study) and copepods which naturally contain HUFA levels appropriate for marine fish larvae (Sargent *et al.*, 1999), must be examined in light of the ongoing prevalence of the jaw malformation in striped trumpeter. Alternatively, early weaning to artificial diets coincident with flexion and prior to the onset of the malformation may be a successful approach if this problem is indeed nutritional.

This study has described development of the cranium and jaw in cultured striped trumpeter larvae prior to flexion and the jaw malformation that proceeds in post-flexion fish. Optimising feeding, growth and survival of striped trumpeter in culture will be dependent upon producing fish without jaw malformation. Further investigation of the culture requirements of this species, including nutrition (essential fatty acids and vitamins), and physical environmental conditions during larval culture (temperature, salinity, light, water quality, and flow dynamics) is needed in order to understand their influence on jaw development, reduce the incidence of jaw malformation and advance this species toward commercial production.

5.6 Acknowledgments

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6. Chapter Six. General discussion and summary.

6.1 General Discussion

6.1.1 Sensory organs

Striped trumpeter larvae are typical of the pelagic larvae of broadcast spawning marine teleosts, in that they hatch at an early stage of development and rely upon endogenous yolk and oil drop reserves until the commencement of feeding. Despite their early stage in development, striped trumpeter larvae have sensory organs potentially able to respond to specific stimuli from an early age (Chapter 2). Mechanoreceptive superficial neuromasts and chemosensory olfactory organs are present at hatching, and development of the visual system occurs rapidly, to become functional coincident with first-feeding on day 7 post-hatching (at 14°C). Startle responses elicited by superficial neuromasts facilitate the escape of larvae from potential predators (Poling and Fuiman, 1997), but may be initiated in culture situations by vibrations, sounds, contact of larvae with tank walls, and the presence of other larvae (Iwai, 1980; Blaxter, 1986). Striped trumpeter developed fewer superficial neuromasts prior to lateral line canal formation than observed in larvae of other species (Blaxter and Fuiman, 1989; Poling and Fuiman, 1997). Larvae associated with complex habitats were found to have more free neuromasts than those in open water, possibly due to their requirement to detect predators and prey in an environment where vision may be limited by obstacles (Poling and Fuiman, 1998). This suggests striped trumpeter are suited to a pelagic existence, which is supported by the fact that vision is the primary sense involved in striped trumpeter feeding, dominating the non-visual sense organs (Pankhurst and Hilder, 1998; present study). In the wild, larval chemosensory abilities assist in their detection of and positioning within prey patches prior to first-feeding (Døving and Knutsen, 1993). However, in intensive culture, prey is often not provided until the onset of feeding, and is generally evenly distributed within the tank such that location of prey via

chemoreception may not be an important factor. Feeding-type behaviours can be elicited by chemical stimuli in yolksac larvae (Knutsen, 1992; Døving and Knutsen, 1993), although chemoreception becomes more important with larval age, as it contributes to feeding behaviour (Dempsey, 1978; Blaxter, 1986). Batty and Hoyt (1995) found juvenile Dover sole were primarily reliant upon chemoreception and mechanoreception for prey detection, while in striped trumpeter the contribution of non-visual senses was lower than that of vision (Chapter 2).

The sensory development of striped trumpeter described in the present study was determined from cultured larvae. It is possible that development in wild larvae may be different, although specimens are not available to confirm this. Kawamura *et al.* (1989) reported differences in the timing and order of sensory organ development in wild and reared Japanese flounder *Paralichthys olivaceus* larvae, although the differences were minimal. Ideally, wild larvae should be used as a benchmark when attempting to achieve 'normal' morphological development in the culture of larvae (Boglione *et al.*, 2001a,b). Blaxter (1988) suggested that sensory deprivation may occur in culture situations, where larvae are not exposed to normal environmental conditions (e.g. gradually changing light intensity at dawn and dusk) and subsequently their behaviour is affected in comparison with fish developing in the wild. While morphological differences between wild and reared larvae may be small, behavioural differences may impede larval growth and survival in culture (Blaxter, 1988).

To avoid the energy expenditure of larvae associated with the startle response and chemically stimulated 'feeding' behaviour, care should be taken to minimise disturbances to the larvae particularly during the period of reliance upon the finite yolk reserves. The energy of older larvae may be replenished once feeding commences, provided they are able to assimilate enough food. Alternative rearing strategies, such as semi-intensive or extensive culture, with lower larval densities and larger tanks/enclosures, should be considered to reduce larval disturbance. These methods have significantly improved the growth and survival

of the larvae of other cultured species (reviewed by Blaxter, 1988; van der Meeren and Naas, 1997).

The feeding performance of the larvae used to examine the relative contribution of non-visual senses in Chapter 2 was relatively low when compared to the larvae used to investigate the effects of light intensity and turbidity (Chapter 4). Consequently, the relative contribution of non-visual senses to larval feeding behaviour should be investigated further through feeding trials and analysis of feeding behaviour. Since low levels of feeding occurred in the dark in the present study (Chapter 2), there may be a benefit in terms of growth and/or survival associated with providing live feed during the dark phase, and this remains to be tested.

The time available for striped trumpeter larvae to feed visually could be extended in culture via manipulating photoperiod to provide a longer or a continuous light phase. Other studies have found the growth of larvae may be improved by long photoperiods (18-h - Barahona-Fernandes, 1979; 19-h at low prey density, 25 or 50 copepod nauplii.ml^{-1} - Dowd and Howd, 1980 or using 24-h light Tandler and Helps, 1985; Duray and Kohno, 1988). Conversely, the survival of larvae may not be as high in 24-h light than with a photoperiod (Barahona-Fernandes, 1979; Ronzani Cerqueira and Chatain, 1991), and continuous light may impede swimbladder inflation (Battaglene and Talbot, 1990; Ronzani Cerqueira and Chatain, 1991; Battaglene *et al.*, 1994). Consequently, the effect of altered photoperiod on larval feeding should be tested in conjunction with other larval performance variables before adopting a particular regime for culture.

6.1.2 Feeding Behaviour

The functional visual field of striped trumpeter larvae under typical clearwater culture conditions was relatively small because the maximum reactive distance (RD) of the larvae to their rotifer prey was 97% of larval standard length (Chapter 3), but this is comparable with RD in the larvae of other species

(Blaxter, 1986). In the horizontal plane, the visual field was forward and laterally directed. Larvae reacted to prey at reactive angles of less than 60° to the orientation of the larvae on day 13 and generally less than 90° on day 17 post-hatching. Given the small size of the area searched for prey, and that the area increases with ontogeny, larvae should be provided with adequate prey density in culture to enable prey encounter, detection and capture sufficient to sustain growth and survival. Hunter (1980) suggested 1 to 4 prey (microcopepods) per ml would generally be suitable for cultured larvae, with prey density requirements declining with larval age. Prey density used in larviculture tends to range from 3-20 rotifers.ml^{-1} in first-feeding and small larvae, and 1-5 *Artemia*.ml^{-1} in older/larger larvae (Ostrowski, 1989; Thomas *et al.*, 1995; Zohar *et al.*, 1995). The current rearing methods with striped trumpeter, using 5 to 10 rotifers.ml^{-1}, are likely sufficient for prey detection in terms of larval visual field. However, in future, the larvae's 3-dimentional search volume and their rates of prey encounter and capture should be examined in relation to larval nutritional requirements.

The declining prey capture success observed in striped trumpeter larvae (Chapter 3) may result from a decline in the health of the larvae, or may indicate a nutritional deficiency compromising the normal development of the neural and retinal structures (Bell *et al.*, 1995). There was no evidence, in the present study, of compromised retinal structures in larvae examined by histology. A decline in prey capture success in cohorts of striped trumpeter could be used to signal declining health and upcoming mortality. However, the observed decline more likely reflected the level of satiation of the larvae, as satiated larvae continue to pursue prey but not complete the strike. Either way, this aspect of larval behaviour should be investigated further since the energy expenditure of larvae in pursuit of prey but not succeeding in capture could be the cause of reduced growth and/or mortality in culture.

The structure of the retina of striped trumpeter larvae revealed the dorsal, dorso-temporal and temporal regions of the retina were most suited to acute image formation, corresponding to a predicted forward and downward directed visual

field (Chapters 2 & 3). This was confirmed in the forward direction by analysis of feeding behaviour in the horizontal plane (Chapter 3), but the functional visual field remains to be examined in the vertical plane. The theoretical acuity of larvae improved with ontogeny, however this was not matched by the functional measures of acuity, which were much poorer and did not change appreciably with age between day 13 and day 17 post-hatching. It has been suggested that the development of higher order neural processing may account for this difference (Pankhurst, 1994). In addition, the functional unit for light capture in the retina is not known, nor the convergence onto ganglion cells, so that the formula of Neave (1984), using the distance between adjacent cones as the finest resolvable unit, underestimates MSA and increases the discrepancy between theoretical and functional measures (van der Meer and Anker, 1984; Browman *et al.*, 1990; Wanzenböck *et al.*, 1996). This means that theoretical acuity does not reliably predict behavioural response, and the actual behaviour of larvae provides a better measure of acuity (Wanzenböck *et al.*, 1996). However, behavioural measures are also influenced by environmental factors, and are open to interpretation regarding the point at which an acute image is formed (Luecke and O'Brien, 1981; Douglas and Hawryshyn, 1990). In the case of striped trumpeter, an acute image of prey may be formed from the RD, or as larval behaviour suggests at the pre-strike distance (PS), or somewhere in between. Nonetheless, RD provides the point at which a prey is detected (if not visually resolved) and is useful for describing the area within which prey may be located.

Since striped trumpeter larvae are primarily visual feeders, there is scope to manipulate RD and feeding success in culture. Reactive distances may be affected by prey characteristics, such as density, size, visibility (contrast), mobility and type (Confer *et al.*, 1978; Wright and O'Brien, 1982; Evans and O'Brien, 1988; Wanzenböck and Schiemer, 1989). Environmental parameters, such as light intensity and turbidity (Vinyard and O'Brien, 1976; Utne, 1997; Utne-Palm, 1999), and predator attributes, including fish size, experience and satiation level (Confer *et al.*, 1978; Breck and Gitter, 1983; Wanzenböck and Schiemer, 1989; Browman and O'Brien, 1992), also influence RD. The present

study of visual field (Chapter 3) has provided baseline data on the functional capabilities of striped trumpeter larvae, against which the effects of a range of culture parameters may be assessed. The influence of light intensity and turbidity have been investigated using short-term feeding trials (Chapter 4), but their effect on the feeding behaviour of the larvae could be quantified in future using the videocinematography methods developed and described in Chapter 3.

6.1.3 Light intensity

Striped trumpeter larvae reared in greenwater fed equally well in clearwater at light intensities in the range 1-10 $\mu mol.s^{-1}.m^{-2}$ (Chapter 4). An improvement in larval feeding performance with age at 0.1 $\mu mol.s^{-1}.m^{-2}$ suggested there was an ontogenetic increase in larval visual sensitivity, supporting the findings of Pankhurst and Hilder (1998). Changes in the larval retinal structure, specifically increased cone ellipsoid diameter (Pankhurst and Hilder, 1998), and double-cone development (Chapter 2), were likely responsible for the functional sensitivity increase. Pankhurst and Hilder (1998) also demonstrated a decline in the proportion of larvae feeding at 28 days post-hatching (7.99 mm SL, Butler, 1995) at 700 lux (cool white fluorescent light ~8.4 $\mu mol.s^{-1}.m^{-2}$). In contrast, there was no decline in feeding of older larvae, day 23 post-hatching (8.73 mm SL), at the highest light intensity tested in the present study (Biolux® fluorescent light 10 $\mu mol.s^{-1}.m^{-2} \equiv 400$ lux). This may have been due to more advanced retinal development, such as the recruitment of functional rods (Butler, 1995), in the older larvae examined by Pankhurst and Hilder (1998). In future, older larvae need to be tested to investigate this shift in feeding performance, and to determine whether a reduced light intensity is required to improve their feeding performance in culture.

These results and those of Pankhurst and Hilder (1998), indicate that the light intensity regimes provided for striped trumpeter larvae in culture should be modified as larvae develop to maintain an appropriate range for feeding. A light intensity between 1 and 10 $\mu mol.s^{-1}.m^{-2}$ is suitable for larvae until at least day 23 (8.73 mm SL), and at this stage intensity could be reduced to 0.1 $\mu mol.s^{-1}.m^{-2}$

without detrimental effects on feeding. Given the change in visual sensitivity of fish larvae with age, short-term trials examining appropriate light conditions for feeding (described in Chapter 4) could be a useful tool in developing culture techniques for other species. The effects of light intensity on larval growth and survival in culture, through factors such as the incidence of swimbladder inflation (Barahona-Fernandes, 1979) and distribution of larvae and prey (Gulbrandsen, 1996), must be considered in conjunction with results of feeding trials when determining the most suitable light environment in culture. It should be noted that in the absence of information about the spectral sensitivity of larval striped trumpeter, natural sunlight or light sources with a broad spectral range are recommended for larviculture (Appendix 2; Boeuf and Le Bail, 1999).

6.1.4 Greenwater

Among the benefits of greenwater culture, the present study has added to the evidence that larval feeding is improved via the altered visual environment in greenwater (Chapter 4), and may enhance the nutritional benefits of having algae in culture tanks (review by Reitan *et al.*, 1997). Since light intensity in the water column of the algal cell-induced turbidity (greenwater) treatments were within the optimal range for larval feeding (Chapter 4), the effects of turbidity on larval feeding performance were attributed to other aspects of the light environment. In greenwater reared larvae, feeding was higher in the high turbidity treatments than in the clearwater and low turbidity treatments. Similar improvements in larval feeding have been found in other studies (Naas *et al.*, 1992; Bristow *et al.*, 1996; Lazo *et al.*, 2000). It is suggested that within the small visual range of the larvae, algal cells scatter light to either illuminate prey from all sides or provide a bright background against which prey contrast is enhanced (Boehlert and Morgan, 1985). However, turbidity treatments adversely affected feeding performance in young clearwater reared larvae, which suggests that culture history and experience of a particular environment contributes to the larval feeding response. It is possible that the light environment during culture may influence the expression of visual pigments in larval fish, and consequently may affect their ability to detect prey under different light regimes, such as clearwater

and greenwater. Thus, culture history should be considered when interpreting results of feeding trials and where possible controls for the environment transition should be included. Miner and Stein (1993) suggested that the interaction between surface light intensity and turbidity will also affect larval feeding. A study over a range of light intensities, particularly low light intensities where the visual capabilities of larvae are approaching the threshold for feeding, would be useful to further examine the effects of light intensity, turbidity and a novel background light environment. In addition, feeding trials with older larvae would enable the identification of an appropriate clearwater light environment for larvae at the end of the greenwater culture period.

Despite culture history influencing feeding performance, this study indicated that algae-induced turbidity provided for equivalent or better feeding in larvae in most cases compared to clearwater, although this was not reflected in differential growth rates of the cohorts cultured in the two environments (Chapter 4). Since feeding was assessed in short-term trials in the present study (0.5 to 2-h), feeding in the culture tanks over longer periods may have been equivalent in clear- and greenwater, such that no differences in growth were detected. The downwelling light intensity provided throughout the greenwater culture tank was within the range for optimal feeding, or higher at the surface in the centre of the tank. Other environmental factors, such as temperature, in each of the cohorts may have confounded larval growth. In addition, aberrant larval behaviour, such as wall-nosing, was reduced in greenwater compared with clearwater, though this remains to be quantified. Thus, further comparisons between growth, survival, and larval performance in long-term clearwater and greenwater culture trials are warranted.

6.1.5 Jaw development and malformation

Jaw development in striped trumpeter was similar to that in other species (Chapter 5). Teeth were not observed in any of the larvae or post-flexion larvae (to day 62 post-hatching) examined in the present study, which suggests striped trumpeter have a prolonged planktivorous phase reliant upon suction and/or

darting. This feature may impact upon weaning success from live food to a formulated diet, and careful observation of the larvae is required to ensure larvae are ingesting any new diet offered.

The jaw malformation in striped trumpeter is different to others described in the literature, particularly in that the jaw elements appear normal in structure but abnormal in position at the first manifestation of the malformation. The maxilla and pre-maxilla were aligned dorso-ventrally rather than posterio-ventrally, and the hyoid arch elements were in an abnormal ventral position, thus causing a permanently locked open jaw. Once the open jaw malformation is established, torsion of muscles around the jaw twist the developing cartilage and bone into further distorted positions, and mechanical damage due to bumping against tank walls may play a role in the final gross morphology of the malformation. Since the malformation occurred well after the onset of feeding, nutritional factors may be a potential cause. Dietary phospholipids, vitamin C deficiency and excess vitamin A have all been related to cranial and skeletal malformations (Kanazawa *et al.*, 1981; Gapasin *et al.*, 1998; Takeuchi *et al.*, 1998). However, subtle changes occurring earlier in development may go undetected and larval quality may be traced back to the egg and critical stages in early embryogenesis/yolksac development (Kawamura *et al.*, 1989; Bromage, 1995; Boglione *et al.*, 2001b; Pittman *et al.*, 2001). Culture environments produce differences in skeletal structures compared with wild fish, and semi-intensive and ‘mesocosm’ rearing techniques have reduced malformations compared with traditional intensive methods (Boglione *et al.*, 2001b). The use of extensive rearing methods remains to be investigated in striped trumpeter, although larval nutrition is the focus of current research and larval malformation will be monitored in nutritional trials.

6.1.6 Constraints on the present study

The present study described the early development of sensory organs to day 26 post-hatching, and determined appropriate light conditions for feeding in young larvae, to day 23 post-hatching. Due to poor larval survival, and the ongoing

development of culture techniques for striped trumpeter, it was not possible to extend the description to the post-flexion larval stage. Specimens to day 62 post-hatching were examined to describe jaw development and to characterise the jaw malformation present in cultured larvae. According to the morphology of the jaw described in this study, feeding in striped trumpeter larvae would not be affected by jaw malformation prior to flexion. Nevertheless, events during the early larval phases may be responsible for the malformation and remain to be investigated. This study provides a limited data set in a species with a protracted larval stage to metamorphosis, around 40 days, and an approximately 9 month paperfish stage before becoming a juvenile. There is a need to investigate development and behaviour in older larvae and post-flexion larvae in order to meet their requirements in the hatchery and nursery phases of aquaculture.

6.2 Conclusion

This study has demonstrated sensory organs are present in larval striped trumpeter from an early age and that they develop rapidly during ontogeny, with vision the primary sense involved in feeding behaviour. The functional visual capabilities of larvae, specifically the area scanned for prey and the range of light intensity suitable for feeding, improved with larval age. In order to meet the requirements of the larvae's changing capabilities, environmental conditions in larval culture tanks, in particular prey density and the light environment, should be adapted for larvae at different stages in development. Algal-cell induced turbidity (greenwater) ameliorates the light conditions in culture tanks, to mimic the low upwelling light intensity relative to downwelling light intensity found in oceanic waters. Turbid conditions can also enhance larval feeding abilities and reduce aberrant behaviour of larvae, making further investigation of this technique worthwhile in the development of culture methods for striped trumpeter. Particular attention should be paid to transitional stages between greenwater and clearwater rearing environments, as this study demonstrated that larval feeding response can be affected by prior visual experience. The normal structure and position of jaw elements in pre-flexion striped trumpeter larvae suggests that their feeding would not be affected by the jaw malformation apparent in older fish.

Three consecutive cohorts of striped trumpeter juveniles were produced in 1999, which was the first time in 10 years of research that this had been achieved, and was the result of a collaborative approach to developing rearing techniques for this species. Light intensities, rearing protocols, and greenwater methods developed during the present study contributed to this success. Studies of the morphological and behavioural development of larvae, such as those in this study, may benefit the determination of culture protocols in other difficult to rear species.

6.3 Summary

- Non-visual senses are available to striped trumpeter larvae from an early age. Superficial neuromasts are present at hatching and are likely to be responsible for predator avoidance responses. A developed inner ear and innervated olfactory organs were present from day 3 post-hatching, enabling horizontal orientation of larvae via mechanoreception and possibly prey detection via chemoreception.

- Lateral line formation had commenced by day 26 post-hatching.

- Retinal development in striped trumpeter was similar to other primarily visually feeding teleost larvae. The retina was at an early stage of development at hatching, but was presumed functional coincident with the onset of feeding on day 7 post-hatching, consisting of a pure single cone retina suited to acute image formation under high light conditions.

- Rod precursor nuclei were first observed in dorsal regions of the retina by light microscopy on day 12-14 post-hatching (~6.5 mm SL, live), and by transmission electron microscopy from day 11 post-hatching (~5.0 mm SL, live), and increased in linear density with larval age.

- Double cone photoreceptors were apparent from day 16 post-hatching (6.8 mm SL, fixed) and were more common in the dorsal than in the ventral retina.

- The timing of rod and double cone appearance was earlier than found in previous studies, and suggests striped trumpeter larvae have the capacity for increased visual sensitivity prior to flexion. Light regimes in culture may require modification to reflect the changes in the larval retina.

- Larval striped trumpeter feed using all senses available to them; 8 ± 3% of streptomycin-treated larvae fed in the dark (chemoreception and inner ear mechanoreception only) and 27 ± 5% of untreated larvae fed in the light (all

senses available). Larval growth and survival may be improved by providing live feed during the dark phase in culture, facilitating feeding 24 hours per day, however this remains to be investigated.

- The dorsal, dorso-temporal, and temporal regions of the retina were suited to the most acute image formation and sensitivity. This corresponded to a forward and downward directed theoretical visual field.

- The functional visual field of striped trumpeter larvae in the horizontal plane was in the forward and lateral direction, confirming theoretical predictions. The visual field increased with age from day 13 to day 17 post-hatching due to an increase in the range of reactive angles utilised by the larvae and due to the increase of maximum reactive distance within that range. The visual field in the vertical plane remains to be determined.

- The maximum reactive distances recorded in non-starved larvae in culture conditions were 5.07 mm and 5.25 mm on days 13 and 17 respectively, which was 97% of larval SL.

- Pre-strike distances were 0.44 mm and 0.46 mm on days 13 and 17 respectively, 8% of larval SL.

- Striped trumpeter larvae displayed a saltatory pattern of prey search behaviour, searching throughout the visual field from a stationary position, before moving and stopping to search again. The larvae often displayed a side-to-side head movement, likely functioning to precisely locate prey before striking at it.

- Estimates of larval visual acuity derived from analysis of feeding behaviour were poorer than those determined from retinal histology, and the estimates ranged from around 2° using maximum reactive distance to close to 40° using pre-strike distance. The latter value may be a closer estimate of the point at which a clear image of the prey item is formed.

- Theoretical visual acuity (MSA) decreased significantly with age from 1°17' of visual arc at day 8 post-hatching (coincident with first-feeding) to 44' arc at day 21 post-hatching, largely due to increasing eye size.

- Larvae reared in greenwater fed equally well in clearwater in a light intensity range of 1-10 $\mu mol.s^{-1}.m^{-2}$, when evaluated in terms of both the proportion of larvae feeding and larval feeding intensity. An ontogenetic improvement in photopic visual sensitivity of larvae was indicated by improved feeding at 0.1 $\mu mol.s^{-1}.m^{-2}$.

- Algal cell-induced turbidity was shown to reduce incident irradiance with depth more rapidly with increasing cell densities (0 to 2 x 10^6 $cells.ml^{-1}$). Light intensities in test chambers used for feeding experiments were within the optimal range for feeding (1-10 $\mu mol.s^{-1}.m^{-2}$), such that observed effects on feeding were not attributed to changes in light intensity but to other properties of the light environment.

- Algae-induced turbidity had different effects on larval feeding response dependent upon the previous visual environment of the larvae. Young larvae (day 9 post-hatching) reared in clearwater showed decreased feeding capabilities with increasing turbidity, while older clearwater reared larvae fed well at all turbidities tested. Greenwater reared larvae had increased feeding capabilities in the highest algal cell densities tested compared with those in low algal cell density, and clearwater to which they were naive. The results raise the possibility that the previous experience of either a clearwater or greenwater visual environment may affect subsequent feeding responses of larvae, and this is an hypothesis that requires further investigation. The transition of larvae from greenwater to clearwater must be managed carefully in larval culture to compensate for prior experience.

- The effect of algae-induced turbidity on larval feeding, where the light intensity in the turbid treatments was within the range for optimal feeding, suggests that turbidity alters feeding response in larvae through its effect on

prey visibility rather than through changes in light intensity. The effect of turbidity on larval feeding behaviour (e.g. reactive distance) remains to be investigated.

- Jaw malformation was only evident in post-flexion larvae greater than 10 mm SL and was characterised by an open jaw in which the maxilla and premaxilla were aligned dorso-ventrally, and the anterior hyoid arch elements were in an abnormal ventral position. X-radiography of the heads of juvenile and adult cultured striped trumpeter revealed ventro-lateral distortion of the jaw elements in comparison with wild-caught fish. It is likely that the feeding performance of pre-flexion larvae is not affected by the jaw malformation, which manifests in post-flexion larvae.

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Appendix One. Vital staining of neuromasts with Janus Green

Method: Vital staining of live striped trumpeter larvae with Janus Green for the observation of free neuromasts

The method employed for the vital staining of striped trumpeter (*Latris lineata*) larvae was modified from the technique used by Blaxter *et al.* (1983) with larval and juvenile herring (*Clupea harengus*). A stock solution of 0.2% Janus Green was made by dissolving 0.2g of stain (Janus Green B, Sigma) in 100ml of distilled water. Larvae were stained in a 0.1% solution of Janus Green, made from 50 ml of stock solution and 50 ml of 5 μm filtered seawater. The stain was made fresh daily from the stock solution, and the stock was discarded after one week. Larvae were collected from culture tanks with a beaker and carefully transferred with a wide-mouthed pipette to a vessel with mesh sides and a solid base within a larger beaker of stain. Five to ten live larvae were stained at a time. The larvae were exposed to the stain for 60 minutes, then the vessel was lifted out of the stain and placed in a 1 litre beaker of 5 μm filtered seawater to rinse off the stain. This method of transferring larvae from the stain to the rinsing water maintained them in at least 10ml of water in the base of the vessel, ensuring the larvae were not exposed to air, an event which results in mortality in striped trumpeter larvae. Turbulence was also minimised with this technique, reducing the possibility of damaging larvae, and particularly of dislodging neuromast cupulae (Blaxter and Fuiman, 1989). Stained larvae were removed from the vessel with a pipette and examined with a stereo microscope to determine the number and position of free neuromasts. The positions of neuromasts were mapped on *camera lucida* drawings of larvae.

The neuromasts stained pale to dark green, with cupulae appearing almost black. Staining intensity developed with time after removal of the larvae from the stain and best results were achieved by observing larvae within 10 to 30 minutes of staining. The intensity of staining was variable within and between striped

trumpeter larvae, which is in agreement with the findings of Blaxter *et al.* (1983). Consequently, neuromast distribution in striped trumpeter larvae was derived from a composite of observations from several larvae of the same age, which is a technique utilised in several other studies (e.g. Blaxter *et al.*, 1983; Higgs and Fuiman, 1996; Poling and Fuiman, 1998).

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Appendix Two. The emission spectra of light sources used in larval rearing

Materials and Methods

A Li-Cor LI-1800 portable spectroradiometer was used to measure the spectral irradiance between 300 and 700nm from a range of light sources used in larval rearing. The light sources tested were sunlight, incandescent spotlights (Philips), cool white fluorescent globes (Crompton), and Biolux® fluorescent globes (Osram) with and without shade cloth.

Results

Sunlight had a high irradiance across the visible spectrum, 300-700nm, increasing from 300nm to a plateau from around 500nm. In contrast, the fluorescent globes tended to peak at particular wavelengths and the Biolux globe had a broader spectral range, particularly from 450-550nm, than the cool white light source. Covering Biolux lights with shade cloth reduced light intensity equally across the emission spectra, such that the wavelength peaks and the spectral composition of the light were preserved. Irradiance from the incandescent globe increased exponentially with increasing wavelength, producing light dominated by the red end of the visible spectrum (Fig.A2.1).

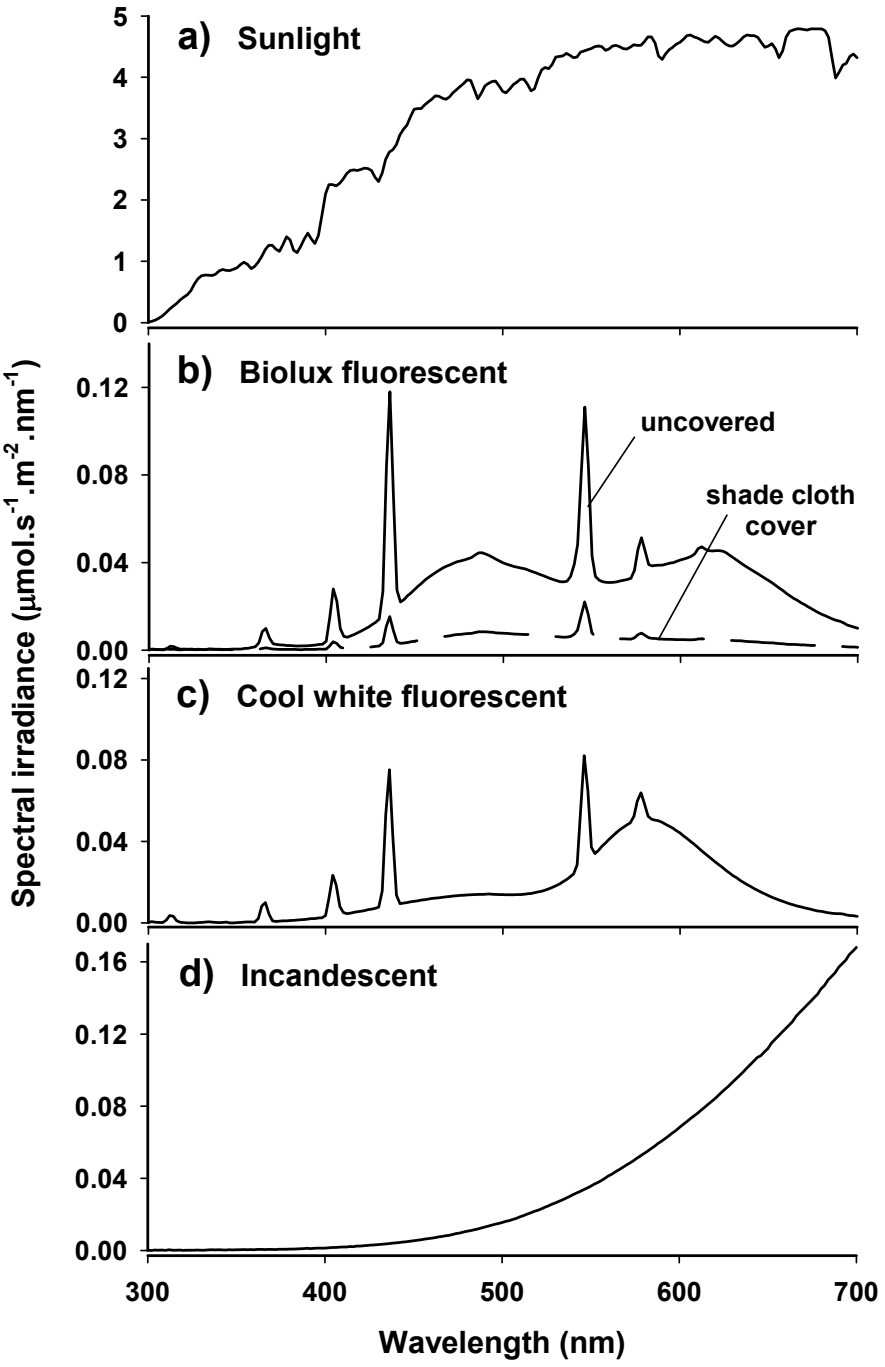

Figure A2.1. Spectral irradiance from a range of light sources measured with a Li-Cor LI-1800 portable spectroradiometer.

Discussion - Provision of artificial light in larval culture

Different light sources affect light intensity and the spectral composition of light underwater, even when they have similar intensities at the surface, because wavelengths are selectively altered underwater (Lythgoe, 1988). The wavelength of maximum transmittance, that is with the least attenuation, in deep blue oceanic water is about 475nm, and in coastal water it is approximately 575nm, and light intensity at that wavelength is depth dependent (Lythgoe, 1988). The colour of water due to dissolved compounds and suspended particles affects the wavelengths of light that are absorbed and those that are scattered and transmitted. The spectral sensitivity of photoreceptors in the retinae of adult and larval fishes has been shown to correspond with predominant wavelengths in their natural environment, and to change during ontogeny corresponding with habitat shifts (Blaxter, 1968; Blaxter, 1969; Lythgoe, 1988). Utne-Palm (1999) demonstrated that the reactive distances of the goby (*Gobiusculus flavescens*) to prey with low contrast relative to the turbid background, were longest at the wavelengths of the fish's spectral sensitivity. Since the spectral sensitivity of particular species is often unknown, 'true-light' tubes with near to natural light composition, such as the Biolux tubes used in this study, are often used in aquaculture (Boeuf and Le Bail, 1999). The emission spectrum from an incandescent globe was red dominated, which could limit the light intensity available at wavelengths preserved under water (green-blue). This may not pose a problem in shallow tanks in clearwater where light attenuation would be minimal, but it would certainly affect light conditions in deeper tanks or with turbid water. Until further research is conducted to determine the spectral sensitivity of larval striped trumpeter, the use of light sources with a broad spectral base is recommended for larval culture.

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Appendix Three. Retinal morphometric data in different regions of the eyes of larval striped trumpeter

The following tables provide the average of photoreceptor cell density, minimum separable angle, and light path length (width of outer segment plus pigment epithelial layers) in the retinae of striped trumpeter larvae from transverse and sagittal sections. The tables provide mean values (± SE) for the total number of replicate counts (*n*) within larval age, and the mean values for each retinal region from combined larval ages. The number of replicate counts varies due to the different number of larvae of each age sectioned, the variable number of replicate counts in eyes of different sizes, and missing counts in retinal areas distorted by the optic nerve and the embryonic fissure. The fundal region was not sampled in the sagittal sections (see Chapter 3, Section 3.3.2 for clarification).

The tables are structured as 3 x 3 matrices within larval age, with the naso-temporal position of the region observed given horizontally, and the dorso-ventral position given vertically. The corresponding retinal regions, defined in Chapter 3 (Fig.3.1), are given in Table A3.1.

Table A3.1. Retinal regions corresponding to the naso-temporal versus dorso-ventral 3 x 3 matrix of retinal position, examined for morphometric parameters, presented in Tables A3.2 to A3.7.

		Naso-temporal position of region		
		Nasal	Medial	Temporal
Dorso-ventral position of region	Dorsal	*Dorso-nasal*	*Dorsal*	*Dorso-temporal*
	Central	*Nasal*	*Fundal*	*Temporal*
	Ventral	*Ventro-nasal*	*Ventral*	*Ventro-temporal*

Table A3.2. Linear cone cell density in different areas of the retinae of striped trumpeter larvae from A, transverse sections and B, sagittal sections.

Larval Age	**Linear cone cell density (cells. 0.1mm^{-1})**					
A: Transverse Sections	mean ± SE	*n*	mean ± SE	*n*	mean ± SE	*n*
Day 8 (3 larvae)	Nasal		Medial		Temporal	
Dorsal	35 ± 6	3	36 ± 2	3	42 ± 3	3
Central	38 ± 1	3	39 ± 3	3	39 ± 1	3
Ventral	38 ± 2	3	34	1	33 ± 1	3
Day 14 (2 larvae)	Nasal		Medial		Temporal	
Dorsal	36 ± 0	2	38 ± 2	2	39 ± 5	2
Central	37 ± 3	2	38 ± 4	2	40 ± 6	2
Ventral	31 ± 3	2			38 ± 2	2
Day 16 (3 larvae)	Nasal		Medial		Temporal	
Dorsal	36 ± 2	6	37 ± 1	6	33 ± 1	2
Central	38 ± 1	6	40 ± 0	5	41 ± 1	2
Ventral	38 ± 2	6	35 ± 1	2	39 ± 1	2
Day 19 (1 larva)	Nasal		Medial		Temporal	
Dorsal	39 ± 3	2	37 ± 1	2	38 ± 2	2
Central	37 ± 3	2	42	1	43 ± 1	2
Ventral	33 ± 1	2	31 ± 3	2	37 ± 3	2
Day 21 (1 larva)	Nasal		Medial		Temporal	
Dorsal	39 ± 1	2	37 ± 1	2	42 ± 2	2
Central	40 ± 0	2	38 ± 0	2	46	1
Ventral	33 ± 1	2	34 ± 2	2		
Average (TS all days)	Nasal		Medial		Temporal	
Dorsal	37 ± 1	15	37 ± 1	15	39 ± 1	11
Central	38 ± 1	15	39 ± 1	13	41 ± 1	10
Ventral	36 ± 1	15	33 ± 1	7	36 ± 1	9
B: Sagittal Sections						
Day 7 (1 larva)	Nasal		Medial		Temporal	
Dorsal	48	1	44	1	48	1
Central						
Ventral	48	1			46	1
Day 12 (1 larva)	Nasal		Medial		Temporal	
Dorsal	46	1	40	1	46	1
Central	46	1			44	1
Ventral	52	1	54	1	42	1
Day 16 (1 larva)	Nasal		Medial		Temporal	
Dorsal	40	1	46	1	40	1
Central	46	1			48	1
Ventral	40	1			44	1
Day 26 (1 larva)	Nasal		Medial		Temporal	
Dorsal	45 ± 3	2	44 ± 3	3	45 ± 3	2
Central	44	1			42 ± 0	2
Ventral	39 ± 3	2	48	1	39 ± 1	2
Average (SS all days)	Nasal		Medial		Temporal	
Dorsal	45 ± 2	5	44 ± 2	6	45 ± 2	5
Central	45 ± 1	3	±		44 ± 1	4
Ventral	44 ± 3	5	51 ± 3	2	42 ± 1	5

Table A3.3. Angular cone cell density in different areas of the retinae of striped trumpeter larvae from A, transverse sections and B, sagittal sections.

Larval Age	**Angular cone cell density (cells.10' visual arc^{-1})**					
A: Transverse Sections	mean ± SE	*n*	mean ± SE	*n*	mean ± SE	*n*
Day 8 (3 larvae)	Nasal		Medial		Temporal	
Dorsal	0.136 ± 0.021	3	0.142 ± 0.009	3	0.166 ± 0.011	3
Central	0.150 ± 0.003	3	0.155 ± 0.012	3	0.153 ± 0.003	3
Ventral	0.150 ± 0.012	3	0.139	1	0.129 ± 0.007	3
Day 14 (2 larvae)	Nasal		Medial		Temporal	
Dorsal	0.174 ± 0.010	2	0.184 ± 0.001	2	0.188 ± 0.013	2
Central	0.178 ± 0.004	2	0.183 ± 0.008	2	0.192 ± 0.018	2
Ventral	0.149 ± 0.006	2			0.185 ± 0.021	2
Day 16 (3 larvae)	Nasal		Medial		Temporal	
Dorsal	0.206 ± 0.008	6	0.212 ± 0.004	6	0.192 ± 0.006	2
Central	0.218 ± 0.007	6	0.231 ± 0.006	5	0.239 ± 0.006	2
Ventral	0.216 ± 0.010	6	0.204 ± 0.006	2	0.227 ± 0.006	2
Day 19 (1 larva)	Nasal		Medial		Temporal	
Dorsal	0.254 ± 0.020	2	0.241 ± 0.007	2	0.248 ± 0.013	2
Central	0.241 ± 0.020	2	0.274	1	0.280 ± 0.007	2
Ventral	0.215 ± 0.007	2	0.202 ± 0.020	2	0.241 ± 0.020	2
Day 21 (1 larva)	Nasal		Medial		Temporal	
Dorsal	0.263 ± 0.007	2	0.250 ± 0.007	2	0.283 ± 0.014	2
Central	0.270 ± 0.000	2	0.256 ± 0.000	2	0.310	1
Ventral	0.223 ± 0.007	2	0.229 ± 0.014	2		
Average (TS all days)	Nasal		Medial		Temporal	
Dorsal	0.202 ± 0.013	15	0.203 ± 0.010	15	0.211 ± 0.014	11
Central	0.209 ± 0.011	15	0.213 ± 0.012	13	0.219 ± 0.019	10
Ventral	0.195 ± 0.010	15	0.201 ± 0.013	7	0.188 ± 0.017	9
B: Sagittal Sections						
Day 7 (1 larva)	Nasal		Medial		Temporal	
Dorsal	0.169	1	0.155	1	0.169	1
Central						
Ventral	0.169	1			0.162	1
Day 12 (1 larva)	Nasal		Medial		Temporal	
Dorsal	0.210	1	0.182	1	0.210	1
Central	0.210	1			0.201	1
Ventral	0.237	1	0.246	1	0.191	1
Day 16 (1 larva)	Nasal		Medial		Temporal	
Dorsal	0.187	1	0.215	1	0.187	1
Central	0.215	1			0.224	1
Ventral	0.187	1			0.206	1
Day 26 (1 larva)	Nasal		Medial		Temporal	
Dorsal	0.304 ± 0.020	2	0.297 ± 0.021	3	0.304 ± 0.020	2
Central	0.297	1			0.283 ± 0.000	2
Ventral	0.263 ± 0.020	2	0.324	1	0.263 ± 0.007	2
Average (SS all days)	Nasal		Medial		Temporal	
Dorsal	0.235 ± 0.030	5	0.241 ± 0.028	6	0.235 ± 0.030	5
Central	0.241 ± 0.028	3			0.248 ± 0.021	4
Ventral	0.224 ± 0.021	5	0.285 ± 0.039	2	0.217 ± 0.020	5

Table A3.4. Acuity (minimum separable angle, MSA) in different areas of the retinae of striped trumpeter larvae calculated from lens radius and cone cell density in A, transverse sections and B, sagittal sections.

Larval Age	**MSA (degrees visual arc)**					
A: Transverse Sections	mean ± SE	*n*	mean ± SE	*n*	mean ± SE	*n*
Day 8 (3 larvae)	Nasal		Medial		Temporal	
Dorsal	1.43 ± 0.20	3	1.33 ± 0.09	3	1.14 ± 0.08	3
Central	1.25 ± 0.02	3	1.22 ± 0.09	3	1.23 ± 0.02	3
Ventral	1.26 ± 0.10	3	1.34	1	1.46 ± 0.08	3
Day 14 (2 larvae)	Nasal		Medial		Temporal	
Dorsal	1.08 ± 0.07	2	1.02 ± 0.01	2	1.00 ± 0.07	2
Central	1.05 ± 0.03	2	1.02 ± 0.05	2	0.98 ± 0.09	2
Ventral	1.25 ± 0.05	2			1.03 ± 0.12	2
Day 16 (3 larvae)	Nasal		Medial		Temporal	
Dorsal	0.91 ± 0.04	6	0.88 ± 0.01	6	0.97 ± 0.03	2
Central	0.86 ± 0.03	6	0.81 ± 0.02	5	0.78 ± 0.02	2
Ventral	0.87 ± 0.05	6	0.92 ± 0.03	2	0.82 ± 0.02	2
Day 19 (1 larva)	Nasal		Medial		Temporal	
Dorsal	0.74 ± 0.06	2	0.77 ± 0.02	2	0.75 ± 0.04	2
Central	0.78 ± 0.07	2	0.68	1	0.67 ± 0.02	2
Ventral	0.87 ± 0.03	2	0.93 ± 0.09	2	0.78 ± 0.07	2
Day 21 (1 larva)	Nasal		Medial		Temporal	
Dorsal	0.71 ± 0.02	2	0.74 ± 0.02	2	0.66 ± 0.03	2
Central	0.69 ± 0.00	2	0.72 ± 0.00	2	0.60	1
Ventral	0.84 ± 0.03	2	0.81 ± 0.05	2		
Average (TS all days)	Nasal		Medial		Temporal	
Dorsal	0.99 ± 0.08	15	0.95 ± 0.06	15	0.93 ± 0.06	11
Central	0.93 ± 0.05	15	0.91 ± 0.06	13	0.91 ± 0.08	10
Ventral	0.99 ± 0.06	15	0.95 ± 0.07	7	1.07 ± 0.11	9
B: Sagittal Sections						
Day 7 (1 larva)	Nasal		Medial		Temporal	
Dorsal	1.11	1	1.21	1	1.11	1
Central						
Ventral	1.11	1			1.16	1
Day 12 (1 larva)	Nasal		Medial		Temporal	
Dorsal	0.89	1	1.02	1	0.89	1
Central	0.89	1			0.93	1
Ventral	0.79	1	0.76	1	0.97	1
Day 16 (1 larva)	Nasal		Medial		Temporal	
Dorsal	1.00	1	0.87	1	1.00	1
Central	0.87	1			0.83	1
Ventral	1.00	1		0	0.91	1
Day 26 (1 larva)	Nasal		Medial		Temporal	
Dorsal	0.62 ± 0.05	2	0.63 ± 0.04	3	0.62 ± 0.05	2
Central	0.63	1			0.66 ± 0.00	2
Ventral	0.71 ± 0.05	2	0.57	1	0.71 ± 0.02	2
Average (SS all days)	Nasal		Medial		Temporal	
Dorsal	0.85 ± 0.10	5	0.83 ± 0.10	6	0.85 ± 0.10	5
Central	0.80 ± 0.08	3			0.77 ± 0.07	4
Ventral	0.86 ± 0.08	5	0.67 ± 0.10	2	0.89 ± 0.09	5

Table A3.5. Linear density of rod precursor nuclei in different areas of the retinae of striped trumpeter larvae from A, transverse sections and B, sagittal sections.

Larval Age	**Linear density of rod precursor nuclei (cells. 0.1mm^{-1})**					
A: Transverse Sections	mean ± SE	*n*	mean ± SE	*n*	mean ± SE	*n*
Day 8 (3 larvae)	Nasal		Medial		Temporal	
Dorsal	0 ± 0	3	0 ± 0	3	0 ± 0	3
Central	0 ± 0	3	0 ± 0	3	0 ± 0	3
Ventral	0 ± 0	3	0	1	0 ± 0	3
Day 14 (2 larvae)	Nasal		Medial		Temporal	
Dorsal	3 ± 3	2	3 ± 3	2	3 ± 3	2
Central	3 ± 3	2	0 ± 0	2	1 ± 1	2
Ventral	0 ± 0	2			0 ± 0	2
Day 16 (3 larvae)	Nasal		Medial		Temporal	
Dorsal	6 ± 2	6	8 ± 1	6	8 ± 0	2
Central	6 ± 2	6	3 ± 2	5	8 ± 0	2
Ventral	6 ± 2	6	4 ± 4	2	1 ± 1	2
Day 19 (1 larva)	Nasal		Medial		Temporal	
Dorsal	10 ± 2	2	8 ± 2	2	10 ± 6	2
Central	9 ± 3	2	8	1	7 ± 1	2
Ventral	1 ± 1	2	2 ± 0	2	5 ± 1	2
Day 21 (1 larva)	Nasal		Medial		Temporal	
Dorsal	8 ± 0	2	8 ± 0	2	8 ± 2	2
Central	4 ± 0	2	3 ± 1	2	8	1
Ventral	6 ± 2	2	3 ± 1	2		
Average (TS all days)	Nasal		Medial		Temporal	
Dorsal	5 ± 1	15	6 ± 1	15	5 ± 2	11
Central	4 ± 1	15	2 ± 1	13	4 ± 1	10
Ventral	3 ± 1	15	3 ± 1	7	1 ± 1	9
B: Sagittal Sections						
Day 7 (1 larva)	Nasal		Medial		Temporal	
Dorsal	0	1	0	1	0	1
Central						
Ventral	0	1			0	1
Day 12 (1 larva)	Nasal		Medial		Temporal	
Dorsal	0	1	4	1	0	1
Central	0	1			0	1
Ventral	0	1	0	1	0	1
Day 16 (1 larva)	Nasal		Medial		Temporal	
Dorsal	2	1	8	1	6	1
Central	0	1			2	1
Ventral	0	1			0	1
Day 26 (1 larva)	Nasal		Medial		Temporal	
Dorsal	14 ± 4	2	25 ± 3	3	14 ± 6	2
Central	14	1			5 ± 3	2
Ventral	12 ± 0	2	2	1	11 ± 1	2
Average (SS all days)	Nasal		Medial		Temporal	
Dorsal	6 ± 4	5	15 ± 5	6	7 ± 4	5
Central	5 ± 5	3			3 ± 2	4
Ventral	5 ± 3	5	1 ± 1	2	4 ± 3	5

Table A3.6. Angular density of rod precursor nuclei in different areas of the retinae of striped trumpeter larvae from A, transverse sections and B, sagittal sections.

Larval Age	Angular density of rod precursor nuclei (cells.10' visual arc^{-1})					
A: Transverse Sections	mean ± SE	*n*	mean ± SE	*n*	mean ± SE	*n*
Day 8 (3 larvae)	Nasal		Medial		Temporal	
Dorsal	0.000 ± 0.000	3	0.000 ± 0.000	3	0.000 ± 0.000	3
Central	0.000 ± 0.000	3	0.000 ± 0.000	3	0.000 ± 0.000	3
Ventral	0.000 ± 0.000	3	0.000 ± 0.000	1	0.000 ± 0.000	3
Day 14 (2 larvae)	Nasal		Medial		Temporal	
Dorsal	0.015 ± 0.015	2	0.015 ± 0.015	2	0.015 ± 0.015	2
Central	0.015 ± 0.015	2	0.000 ± 0.000	2	0.005 ± 0.005	2
Ventral	0.000 ± 0.000	2			0.000 ± 0.000	2
Day 16 (3 larvae)	Nasal		Medial		Temporal	
Dorsal	0.033 ± 0.010	6	0.045 ± 0.009	6	0.047 ± 0.000	2
Central	0.033 ± 0.011	6	0.019 ± 0.009	5	0.047 ± 0.000	2
Ventral	0.033 ± 0.012	6	0.023 ± 0.023	2	0.006 ± 0.006	2
Day 19 (1 larva)	Nasal		Medial		Temporal	
Dorsal	0.065 ± 0.013	2	0.052 ± 0.013	2	0.065 ± 0.039	2
Central	0.059 ± 0.020	2	0.052	1	0.046 ± 0.007	2
Ventral	0.007 ± 0.007	2	0.013 ± 0.000	2	0.033 ± 0.007	2
Day 21 (1 larva)	Nasal		Medial		Temporal	
Dorsal	0.054 ± 0.000	2	0.054 ± 0.000	2	0.054 ± 0.014	2
Central	0.027 ± 0.000	2	0.020 ± 0.007	2	0.054 ± 0.000	1
Ventral	0.040 ± 0.014	2	0.020 ± 0.007	2		
Average (TS all days)	Nasal		Medial		Temporal	
Dorsal	0.031 ± 0.007	15	0.034 ± 0.007	15	0.033 ± 0.010	11
Central	0.027 ± 0.007	15	0.014 ± 0.005	13	0.025 ± 0.008	10
Ventral	0.020 ± 0.007	15	0.016 ± 0.006	7	0.009 ± 0.005	9
B: Sagittal Sections						
Day 7 (1 larva)	Nasal		Medial		Temporal	
Dorsal	0.000	1	0.000	1	0.000	1
Central						
Ventral	0.000	1			0.000	1
Day 12 (1 larva)	Nasal		Medial		Temporal	
Dorsal	0.000	1	0.018	1	0.000	1
Central	0.000	1			0.000	1
Ventral	0.000	1	0.000	1	0.000	1
Day 16 (1 larva)	Nasal		Medial		Temporal	
Dorsal	0.009	1	0.037	1	0.028	1
Central	0.000	1			0.009	1
Ventral	0.000	1			0.000	1
Day 26 (1 larva)	Nasal		Medial		Temporal	
Dorsal	0.094 ± 0.027	2	0.171 ± 0.020	3	0.094 ± 0.040	2
Central	0.094	1			0.034 ± 0.020	2
Ventral	0.081 ± 0.000	2	0.013	1	0.074 ± 0.007	2
Average (SS all days)	Nasal		Medial		Temporal	
Dorsal	0.040 ± 0.024	5	0.095 ± 0.036	6	0.043 ± 0.025	5
Central	0.031 ± 0.031	3			0.019 ± 0.012	4
Ventral	0.032 ± 0.020	5	0.007 ± 0.007	2	0.030 ± 0.018	5

Table A3.7. Light path length, width of photoreceptor outer segment (OS) and pigment epithelial (PE) layers, in different areas of the retinae of striped trumpeter larvae from A, transverse sections and B, sagittal sections.

Larval Age	**Light path length (OS + PE) (μm)**					
A: Transverse Sections	mean ± SE	*n*	mean ± SE	*n*	mean ± SE	*n*
Day 8 (3 larvae)	Nasal		Medial		Temporal	
Dorsal	11.6 ± 0.8	9	12.9 ± 0.4	9	12.2 ± 0.7	9
Central	8.9 ± 0.3	9	9.7 ± 0.3	9	9.8 ± 0.5	9
Ventral	7.9 ± 0.3	9	8.7 ± 0.3	3	8.7 ± 0.5	9
Day 14 (2 larvae)	Nasal		Medial		Temporal	
Dorsal	20.2 ± 2.2	6	18.7 ± 1.4	6	18.7 ± 0.6	6
Central	15.7 ± 1.2	6	16.0 ± 0.9	6	16.8 ± 1.7	6
Ventral	13.2 ± 1.1	6			10.8 ± 1.7	6
Day 16 (3 larvae)	Nasal		Medial		Temporal	
Dorsal	21.2 ± 1.1	9	20.1 ± 1.0	9	18.7 ± 0.9	3
Central	17.9 ± 0.6	9	18.6 ± 1.0	9	18.3 ± 1.2	3
Ventral	15.3 ± 0.7	9	16.7 ± 0.3	3	19.3 ± 0.3	3
Day 19 (1 larva)	Nasal		Medial		Temporal	
Dorsal	26.7 ± 2.6	3	22.3 ± 0.7	3	20.7 ± 0.7	3
Central	19.3 ± 0.7	3	18.7 ± 0.3	3	20.3 ± 0.9	3
Ventral	13.7 ± 0.3	3	13.7 ± 0.3	3	13.7 ± 1.2	3
Day 21 (1 larva)	Nasal		Medial		Temporal	
Dorsal	29.3 ± 0.7	3	27.3 ± 0.3	3	27.0 ± 4.0	3
Central	20.7 ± 2.3	3	23.0 ± 2.0	3	27.7 ± 0.3	3
Ventral	15.7 ± 0.9	3	16.7 ± 0.3	3		
Average (TS all days)	Nasal		Medial		Temporal	
Dorsal	19.5 ± 1.3	30	18.6 ± 0.9	30	17.5 ± 1.1	24
Central	15.2 ± 0.9	30	15.8 ± 0.9	30	16.2 ± 1.3	24
Ventral	12.5 ± 0.7	30	13.9 ± 1.0	12	11.5 ± 1.0	21
B: Sagittal Sections						
Day 7 (1 larva)	Nasal		Medial		Temporal	
Dorsal	14.8 ± 2.3	2	17.2	1	19.5 ± 2.3	2
Central						
Ventral	9.4 ± 0.0	2			10.2 ± 2.3	2
Day 12 (1 larva)	Nasal		Medial		Temporal	
Dorsal	15.6 ± 1.6	2	17.2	1	25.0 ± 4.7	2
Central	15.6	1			21.9	1
Ventral	9.4 ± 1.6	2	10.9	1	14.1 ± 0.0	2
Day 16 (1 larva)	Nasal		Medial		Temporal	
Dorsal	10.9	1	23.4	1	23.4 ± 1.6	2
Central	14.1	1			20.3	1
Ventral	11.7 ± 0.8	2	9.4	1	17.2 ± 0.0	2
Day 26 (1 larva)	Nasal		Medial		Temporal	
Dorsal	20.3 ± 1.6	2	33.6 ± 2.3	2	32.0 ± 0.8	2
Central	17.2	1			32.0 ± 2.3	2
Ventral	16.4 ± 0.8	2	15.6 ± 1.6	2	23.4 ± 1.6	2
Average (SS all days)	Nasal		Medial		Temporal	
Dorsal	16.1 ± 1.4	7	25.0 ± 3.8	5	25.0 ± 2.0	8
Central	15.6 ± 0.9	3			26.6 ± 3.3	4
Ventral	11.7 ± 1.1	8	12.9 ± 1.7	4	16.2 ± 1.9	8