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Emmetropisation in the Camera-Type Eye of the Squid

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A thesis submitted in partial fulfilment of the requirements for the degree of
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Abstract

Aim

To establish whether the convergently evolved, camera-type eye of the squid can regulate its eye growth when the focal plane is manipulated by exploiting inherent longitudinal chromatic aberration.

Methods

Sepioteuthis australis squid were separated into two tanks (orange and blue). Each tank was covered with a spectral filter, which restricted wavelengths to either end of the squid spectral sensitivity, and created a longer focal length in the orange tank compared to the shorter focal length in the blue tank. Measurements of relative eye size (Matthiessen's ratio, MR) and refraction (with infrared photorefraction) were compared between squid from each tank at 45 days post-hatching. The spectral filters were switched, and the effecting of modifying the focal length assessed at 60 days post-hatching. Visual acuity (in cycles per degree, CPD) was assessed behaviourally in octopus, and estimated from the squid crystalline lens MTF.

Results

At 45 days post-hatching, squid eyes from the orange spectral environment had a larger MR than those from the blue tank ($p = 0.006$). Relative to infrared light, the squid eyes from the blue tank were more hyperopic than those from orange ($p = 0.004$). When the filters were exchanged, both MR and refraction values for the blue and orange spectral environments also reversed. There was no significant change in growth of either the lens or the retina alone. Visual acuity is high in the octopus (12.23 ± 1.61 CPD) and the squid (6.56 ± 1.14 CPD)

Conclusions

Squid eyes possess an emmetropisation mechanism, and are able to regulate their eye growth by adjusting the relative size of the retina to the size of their lens. Refraction values support the idea that this growth attempts to minimise refractive error. As the squid eye has convergently evolved, this suggests that emmetropisation has evolved at least twice within the Animal Kingdom. As the squid retina is comparatively simple, this may suggest that emmetropisation can function without the complexity of the vertebrate retina.
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I also need to acknowledge Dr Steve O’Shea, who came on board despite his own advice against keeping cephalopods in a laboratory, and donated his time and knowledge to teach me how establish the squid aquarium, find prey for the squid, and how to tend to the octopus.

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List of abbreviations

MYA  millions of years ago
LCA  longitudinal chromatic aberration
TCA  transverse chromatic aberration
DNA  deoxyribonucleic acid
CL   continuous lighting
FDM  form deprivation myopia
UV   ultraviolet
IR   infrared
D    dioptr (m⁻¹)
IS   inner segment
OS   outer segment
OAB  over accommodation to blue
GAG  glycosaminoglycan
ARVO Association for Research in Vision and Ophthalmology
DML  dorsal mantle length
Dₚ  diameter of a circle fitted to the squid retina
Dₗ  diameter of a circle fitted to the squid crystalline lens
Xᵣ, Yᵣ  (X,Y) position of the centre of a circle fitted to the squid retina
Xₗ, Yₗ  (X,Y) position of the centre of a circle fitted to the squid crystalline lens
MR   Matthiessen’s ratio
LED  light emitting diode
ANOVA analysis of variance
MWU  Mann-Whitney u-test
GLM  generalised linear model
CI   confidence interval
LCD  liquid crystal display
CPD  cycles per degree
MTF  modulation transfer function
OBO  a cohort of squid in experiment 3 which moved from the orange, to the blue, then to the orange tank
WOB  a cohort of squid in Experiment 3 which moved from a white (unfiltered) tank, to the orange, then to the blue tank
BBB  a cohort of squid in Experiment 3 which remained in the blue tank for the three periods
ppm  parts per million
SG   specific gravity
RODI reverse osmosis, de-ionized water
TDS  total dissolved solids
ICOLLs intermittently closed and open lakes and lagoons
Chapter 1. Overview

‘Simple’ or camera-type eyes, which possess both focal and aperture control, are often considered the pinnacle of eye evolution and are present in a wide range of vertebrates, from fish to primates. Despite the seemingly paradoxical classification as a simple eye, their single-chambered optical arrangement with one entrance pupil allows much higher visual acuity in a more compact design than that of ‘Compound’ eye types found in many invertebrates. However, the design of the eye alone does not guarantee high acuity: to permit maximum acuity the photoreceptor array must also be conjugate with the image plane created by the eye’s refractive components.

As the eye grows, both the refractive power and the separation between the focal components (cornea, lens, and retina) change, presenting a challenge to maintaining focus, and thus high acuity, throughout the growing phase. The process which keeps the components in focus throughout growth is termed emmetropisation; a failure of this mechanism leads to ametropia. For largely unknown reasons, ametropias, in particular human myopia, are increasing in prevalence and severity in developed countries. The increasing prevalence and the associated socio-economic and medical burdens of ametropias provide a strong impetus to better understand the emmetropisation process itself. However, despite extensive study of numerous vertebrate models of ametropia, we are yet to elucidate the underlying cellular mechanisms. Many studies have shown that vertebrate emmetropisation depends upon a local, optically guided feedback loop that adjusts the position of the retina to match the focal plane created by the optical components. However, the neural intricacies of the vertebrate retina and the optical complexity of the vertebrate eye have hindered studies attempting to understand the emmetropisation mechanism.

The squid eye faces the same optical challenges as the vertebrate eye, yet offers distinct advantages over existing vertebrate models. Squid are highly visual predators, whose eye size increases at least an order of magnitude during their short lifespan. Squid hunt live prey from a few days post-hatching, which suggests an active emmetropisation mechanism would be required from an early age. The cephalopod emmetropisation model is comparatively simple;
their aquatic environment effectively nullifies the refractive power of the cornea, reducing the focal components to a simple spherical lens and hemispherical retina. The cephalopod retina is also very simple, containing only photoreceptors which project directly out of the eye.

The first part of this thesis aims to investigate emmetropisation in the invertebrate camera-type eye of squid (Phylum: Cephalopoda), which has convergently evolved alongside the vertebrate eye. To achieve this, we exploit the phenomenon of longitudinal chromatic aberration (LCA). During refraction of light, shorter (blue) wavelengths are refracted to a higher degree than longer (red) wavelengths. In this study, the presence of emmetropisation is tested by raising squid in different spectral environments - one biased towards blue, one towards red, and switching animals between these spectral environments. We hypothesise that if an optically guided emmetropisation process is present, squid raised in blue light will have more hyperopic refractions (and possibly shorter eyes) than those raised in red light. In addition, the refractive status of animals should change appropriately as they are switched between the different spectral environments.

The second part of this thesis investigates cephalopod visual acuity. An animal with poor visual acuity would likely benefit less from emmetropisation, whereas high acuity would suggest a need for emmetropisation. Octopus, which are also cephalopods, were used to obtain psychophysical measurements of visual acuity. By utilising a novel response of the eye when presented with a new visual stimulus, a decreasing method of limits protocol allowed an estimate of visual acuity. Squid visual acuity was assessed in-vitro by measuring the modulation transfer function of their lens.

The main results of this study indicate that an optically guided emmetropisation process is present in the high acuity cephalopod eye. Based on the independent evolutionary history of vertebrate and invertebrate eyes from a common ancestor without camera-type eyes, it is suggested that optically guided emmetropisation must have evolved independently at least twice. It also appears that in cephalopods, emmetropisation is either regulated by the photoreceptor cells themselves, or encoded in the photoreceptor action potential outputs.
Chapter 2. Emmetropisation

2.1. Emmetropia versus ametropia

The traditional definition of emmetropia describes an eye in which distant light is accurately focused on the retina while the accommodative system is at rest. An eye in which the focal point and retina are misaligned is ametropic. This definition works well for primates, however it is not necessarily true of all animals. Bony fish negatively accommodate; their accommodative system is exerting effort to see in the distance, and is more relaxed at near (Khorramshahi, Schartau et al. 2008), which complicates the definition. When viewing a distant object, the degree of misalignment between the focal plane of the eye’s optics and the retina is described as the refractive error. If the refractive components are too powerful causing the focal plane to be anterior to the retina, the eye is said to be myopic. Conversely, if the refractive components are too weak (focal plane behind the retina), the eye is said to be hyperopic.

2.2. Disadvantages of ametropia

2.2.1. Evolutionary perspective

In evolutionary terms, at the level of the organism, an individual with an ametropia is competitively disadvantaged because they are unable to resolve spatial frequencies as high, nor contrast at lower spatial frequencies as high as their emmetropic competitors (Liou and Chiu 2001). This may translate to an inability to identify a predator in the distance, or the inability to spot fruit amongst leaves on a tree. This suggests that there would be strong evolutionary pressure at an individual level towards achieving and maintaining emmetropia.

There is also pressure at an organ level. The eye and its associated neural processing is one of the most metabolically active tissues in the body (Kaufman, Alm et al. 2003), and there are examples of extant animals demonstrating actively selected regressive evolution by losing their sense of vision when moving into dark environments such as caves (Tobler, Coleman et al. 2010; Wilkens 2010; Klaus, Mendoza et al. 2013) in an effort to conserve energy. While the
metabolically expensive neural processing no longer functions, some of the less energy expensive eye structures remain (Wilkens 2007)).

To maximise energy efficiency of the eye, retinal sampling frequency should not exceed the optical quality of the retinal image. There is strong evidence throughout the animal kingdom that retinal resolution closely follows, or where a discrepancy exists, slightly lags that of the optical resolution limit of the refractive components (Seyer 1994; Artal, De Tejada et al. 1998; Coletta, Marcos et al. 2003; Nilsson, Gislén et al. 2005; Williams 2011). In emmetropic humans, foveal resolution is limited by photoreceptor spacing (Rossi and Roorda 2010). With increasing ametropia the retinal resolution soon exceeds the optical spatial resolution (Xu, Bradley et al. 2013), and maintaining many densely packed retinal cells giving high spatial resolution becomes redundant and wasteful of energy. Consequently, from an evolutionary perspective, it would be expected that ametropias would be selected against.

2.2.2. Anthropomorphic perspective

While human eyes ostensibly possess an emmetropisation mechanism (Flitcroft 2013), in recent times it appears to be failing. Myopia has reached epidemic levels in some East Asian countries (Park and Congdon 2004; Woo, Lim et al. 2004; Leo and Young 2011). The vast majority of myopia is due to the axial length of the eye being too long, rather than the refractive components being too powerful (Carney, Mainstone et al. 1997; Du, Zhang et al. 2013). In modern society, the visual disability can be corrected with ophthalmic lenses or surgery, although there is a significant economic cost (Lim, Gazzard et al. 2009). However, because of the physical elongation of the eye, the tissue layers that comprise the ocular coat (the retina, choroid, and sclera) can become stretched, thinned, and fragile, ultimately leading to potentially blinding pathological conditions such retinal detachment, myopic maculopathy, and glaucoma (Saw, Gazzard et al. 2005; Zejmo, Formińska-Kapuścik et al. 2009). As myopia prevalence and severity has increased, there is already evidence of increasing visual morbidity within the Chinese population (Zou, Zhang et al. 2002). Furthermore, surgery on eyes with substantial myopia have poorer outcomes (Yokoi, Moriyama et al. 2013), and higher complication rates (Haug and Bhisitkul 2012) than surgery on emmetropic eyes. Despite the dramatic increase in errors of emmetropisation, our understanding of the mechanisms underlying emmetropisation remains poor.
2.3. The challenge of eye growth

As an eye grows, the radius of curvature (and therefore optical power) of the cornea and crystalline lens decreases, and the relative position of these elements shift in relation to the retina. This provides a challenge for maintaining emmetropia.

Eye growth has been well studied in humans. At birth, ocular axial length (measured from the anterior corneal surface to the retina) is approximately 17mm (Gordon and Donzis 1985). Eye length increases throughout childhood (Logan, Shah et al. 2011), stabilising near 24mm in adulthood (Foster, Broadway et al. 2010). This increase in axial length is accounted for by increases in both the anterior and vitreous chambers, with corresponding decreases in corneal and lens power (Zadnik, Manny et al. 2003; Mutti, Mitchell et al. 2005).

The tolerance for the balance between the separate components in an individual eye is low. In an adult eye, misalignment of approximately 350 microns between the focal plane and the retina (about 1.4% of the total axial length) causes approximately one dioptre of refractive error (Norrby 2008). With a 5mm pupil, this small misalignment halves distance acuity from 6/6 to 6/12. As misalignment of any of the individual components (e.g. the crystalline lens) will cause ametropia, this raises the question of how the growth and relative positions of the individual components is coordinated to maintain emmetropia as the eye grows.

2.4. Distribution of refractive error in humans

A system that ensures accurate alignment of the focal plane and the retina could occur through chance, be genetically pre-programmed, or be visually guided. Early work by Sorsby et al. (Sorsby, Leary et al. 1962) on a large number of human eyes showed that each component of refraction; namely the corneal curvature, lens power and spacing of the components (anterior and vitreous chamber depths) individually have Gaussian distributions, as expected. At birth, when an infant has very little visual experience, the distribution of refractive errors is also a Gaussian distribution with a slightly hyperopic mean (Brown, Koretz et al. 1999). During the first few years of life, this distribution changes; the variance decreases significantly, and the shape changes to a leptokurtic distribution tightly centred on low hyperopia (Brown, Koretz et al. 1999). Ametropias between about -4.00 and +6.00D could be attributable to an error of
correlation of the individual ocular components, while refractive errors outside this range are usually attributable to an abnormal single component, typically ocular axial length (Sorsby 1956). For a population of emmetropic eyes, there is wide variation in the values of the individual components among eyes (Stenström 1948), yet all eyes are accurately focused. This provides epidemiological evidence that there is not a normal biological distribution, nor ideal parameters of eye lengths and refractive powers, which just happen to align some of the time, but rather an active process which coordinates the growth of the individual optical components of a disproportionally large number of eyes towards emmetropia (Sorsby 1956).

The shift from predominantly hyperopic refractions towards emmetropia in the years after birth is not unique to humans: it has also been observed in wide range of vertebrates, including monkeys (Hung, Crawford et al. 1995), chicks (Wallman, Adams et al. 1981), kestrels (Andison, Sivak et al. 1992), tree shrews (Norton and McBrien 1992), mice (Schmucker and Schaeffel 2004), and guinea pigs (Zhou, Qu et al. 2006).

2.5. Emmetropisation in vertebrates

Studying the physiological mechanisms underlying emmetropisation is difficult in humans, on both ethical and practical grounds. Using unproven interventions over the developmental period is difficult and carries high risk, both in terms of interference with education, and the potential for negative outcomes for the remainder of the subject’s life. Interventional studies also require significant time, which adds to the cost. In addition, sampling the tissues of interest, namely the retina, choroid and sclera, is not currently possible in vivo, which limits the types of outcomes that can be measured. Therefore animal models have been developed in an attempt to understand the mechanisms of emmetropisation.

2.5.1. Natural emmetropisation in vertebrates

Emmetropisation appears to function well in the animal kingdom, and ametropia is almost exclusively a human affliction. However, spontaneous axial myopia has been reported in a strain of lab guinea pig (Jiang, Schaeffel et al. 2009). Myopia also occurs in some species of dogs (Murphy, Zadnik et al. 1992; Black, Browning et al. 2008; Kubai, Bentley et al. 2008). In some
canine species, myopia appears to be refractive rather than axial in nature (Williams, Kubai et al. 2011), although increased vitreous chamber depth appears to be the cause of myopia in Labrador Retrievers (Mutti, Zadnik et al. 1999). However, dogs are generally highly domesticated, often constrained in artificial environments, and poorly represent their wild brethren.

Selective breeding of chicks on the basis of the most and least responsive to form deprivation myopia, over as few as three generations, results in a significant difference between baseline refractions, although neither group was myopic (Chen, Hocking et al. 2011). It remains to be seen whether further selective breeding can induce the comparatively spontaneous myopia seen in humans.

2.5.2. Manipulating emmetropisation in vertebrates

With poor spontaneous analogues of human refractive error, animal models are created by modulating the inputs to emmetropisation. In order to use an animal model, refractive error first has to be induced, usually despite a functioning emmetropisation mechanism and this may be different to the human condition (Zadnik and Mijtti 1995). However the physical elongation of the vitreous chamber seen in animal models appears similar to that in human myopia (Flitcroft 2013).

Myopia can be induced in animals by keeping them in a restricted environment (Young 1963; Belkin, Yinon et al. 1977), or by manipulating light entering the eye, both of which have interesting implications for the modern humans’ increasingly urbanised lifestyle. Models of myopia have been made in mice (Barathi, Boopathi et al. 2008), rats (Mutti, Zadnik et al. 1998), rabbits (Bryant, Kampmeier et al. 1999), cats (Rose, Yinon et al. 1974), guinea pigs (Howlett and McFadden 2006), chicks (Harrison, Bercovitz et al. 1968), monkeys (Von Noorden and Crawford 1978; Criswell and Goss 1983; Tigges, lvone et al. 1999), squirrels (McBrien, Moghaddam et al. 1993), and tree shrews (Sherman, Norton et al. 1977; Cottriall and McBrien 1996). All are homoeothermic vertebrates, and all but chicks, are mammals. In 2005, a group from Waterloo (Shen, Vijayan et al. 2005) were successful in inducing myopia in Tilapia, a type of cichlid fish, and the most evolutionary distant vertebrate model from humans. Many animal studies show high variability in response to an apparently identical stimulus, both between species and
between individuals in the same study, which complicates the extrapolation of experimental conclusions throughout Animalia (Zadnik and Mijtti 1995).

2.6. Emmetropisation inputs

Emmetropisation research can be broadly divided into three investigative categories: inputs affecting emmetropisation, the anatomical location of the system, and the chemical or neural signalling of the system. As this thesis investigates the presence of an emmetropisation mechanism by manipulating light entering the eye, the following is a review of inputs that can interfere with emmetropisation in vertebrates.

2.6.1. Light exposure

Light is required for achieving emmetropisation (Guiton, Greene et al. 1989), and for maintenance of emmetropia during growth after initial emmetropisation has been achieved (Norton, Amedo et al. 2006). Experiments that prevent light from entering the eye (such as dark rearing) are said to be ‘open loop’ experiments in that they prevent external input to the emmetropisation feedback loop (Troilo, Nickla et al. 2000). The effect is often unpredictable; occasionally it may produce excess ocular elongation (e.g. tree shrews (Norton, Amedo et al. 2006)), little change (guinea pigs (Howlett and McFadden 2006)), or tends towards hyperopia (chicks (Troilo 1990)). However, it may be hard to predict the effect of darkness on other essential attributes, such as feeding and exercise, or how much of the abnormal growth can be attributed to a disrupted circadian rhythm (Lauber and Oishi 1989). Similarly, continuous lighting (CL) also produces results which vary by species. In chicks, CL appears to interfere with anterior eye development, creating hyperopic eyes with flat corneas and short anterior chambers (Li, Howland et al. 2000). In rhesus macaques, CL appears to have minimal effect on normal emmetropisation (Smith III, Bradley et al. 2001).

2.6.2. Form deprivation

Emmetropisation under normal lighting conditions can be disrupted by preventing the formation of a high spatial frequency image on the retina. This can occur naturally in humans, as
in the case of congenital cataracts or corneal opacity (Rabin, Van Sluyters et al. 1981; Meyer, Mueller et al. 1999), or artificially in animals through the use of a diffuser lens worn in front of the eye (Gottlieb, Joshi et al. 1990; Cottriall and McBrien 1996; Smith III, Hung et al. 2002). In all animals studied to date, form deprivation causes abnormal axial elongation of the eye and consequently myopia. If the region of form deprivation is restricted to a region of the retina, only the occluded area grows abnormally in both chicks (Wallman, Gottlieb et al. 1987) and rhesus macaques (Smith III, Huang et al. 2009), suggesting a regional defocus detection and response mechanism within the eye. Additionally, in both chicks (Troilo, Gottlieb et al. 1987) and rhesus macaques (Raviola and Wiesel 1985), axial elongation of the eye still occurs in response to form deprivation after optic nerve section, suggesting local control of ocular growth. The form deprivation model may suggest that in the absence of focus, the default state of the eye is to grow, and that emmetropisation merely acts as a brake in a uni-directional system, as seen with the rapid recovery of the induced myopia when the diffuser is removed.

2.6.3. Optical defocus

The effect of optical defocus is generally investigated by manipulating the position of the focal plane with spectacle lenses or contact lenses. An advantage of this method is that both hyperopic (with negative lenses) and myopic defocus (with positive lenses) can be simulated, allowing the bi-directionality of emmetropisation to be tested. If the eye compensates for the power of the lens, then when the lens is removed the eye will either be myopic (after removal of negative lens) or hyperopic (after removal of a positive lens). Similarly to form deprivation, all animals studied so far are able to overcome negative lens induced defocus (simulating myopia), although the commensurate range varies significantly by species (Smith III 2011). When lenses from +6.00D to -6.00D are introduced in front of a rhesus monkey eye, a close relative of humans, the eye grows to neutralise the induced refractive error (Smith III and Hung 1999). Marmosets, a more distantly related primate, when fitted with positive or negative power daily contact lenses grow to reduce the ametropia between ±4.00D (Whatham and Judge 2001). Guinea pigs show an asymmetrical response to lens wear, responding to negative lenses up to -4.00D by increasing ocular axial length, however the ocular response to positive lenses are less pronounced (Howlett and McFadden 2009). The chick eye demonstrates a wider range of recoverable ametropia, and is typically able to regulate eye growth to overcome induced error
between +10.00 and -15.00D, largely by adjusting the rate of axial growth, although corneal flattening plays a role when the eye is made functionally hyperopic with convex lenses (Irving, Sivak et al. 1992).

How the eye determines the sign and magnitude of imposed defocus, and stops the compensatory process once emmetropia is achieved is not known. However, the tolerable range of emmetropia appears to be shared between the eyes (Tepelus and Schaeffel 2010). There is some evidence in tree shrews that the sign, but not the magnitude, of defocus guides the emmetropisation response (Amedo and Norton 2012). However, the regulation of eye growth appears to be largely local to each individual eye. In chicks, normal emmetropisation (with no lens) is disrupted after optic nerve section (Troilo, Gottlieb et al. 1987; Wildsoet and Pettigrew 1988). However, the chick eye still responds to lens induced defocus appropriately, despite sectioning of the optic nerve, although it may lack an element of refinement provided by higher neural centres (Wildsoet and Wallman 1995). If a portion of the retina (e.g. nasal or temporal) is exposed to defocus, only the affected portion demonstrates a compensatory response to the lens defocus (Diether and Schaeffel 1997). When competing hyperopic and myopic defocus is simultaneously presented to the retina with a concentric dual focus lens, the eye compensates for a mean of the two powers, with a slight bias towards the myopic retinal defocus in both chicks (Tse, Lam et al. 2007) and marmosets (Benavente-Perez, Nour et al. 2012). Adjusting the proportional area of alternating myopic and hyperopic defocus (with +10.00/-10.00D), causes the chick retina to grow towards a weighted mean of the imposed defocus (Tse and To 2011).

Humans have demonstrated a reduced rate of ocular elongation after wearing a dual focus lens, designed to create simultaneous emmetropia and +2.00D dioptre myopic defocus on the retina (Anstice and Phillips 2011).

In addition to spatial summation, temporal summation of defocus plays a role in emmetropisation (Zhi, Pan et al. 2013). The initiation of an emmetropisation response to imposed defocus can occur in a matter of minutes (Zhu, Park et al. 2005; Read, Collins et al. 2010), but the response persists for much longer than the exposure period (Zhu and Wallman 2009). When chicks are exposed to as little as two minutes of hyperopic defocus during the day, the response of the eye after three days of exposure is almost as strong as if the lenses had been worn continuously (Winawer and Wallman 2002). Guinea pigs also show a strong response to short periods of hyperopic defocus which, in the absence of other focal cues, can be effectual
over a substantially longer period than the applied defocus (Leotta, Bowrey et al. 2013). When myopic defocus is alternately presented, only a comparatively short period of myopic defocus is required to negate the effects of hyperopic defocus (Zhu, Winawer et al. 2003; McBrien, Arumugam et al. 2012). Ocular length elongation in response to hyperopic defocus or form deprivation can also be reduced by providing brief periods of uninterrupted distance vision (Smith III, Hung et al. 2002; Kee, Hung et al. 2007).

Light intensity also appears to have an effect on eye growth. When lens myopia is induced in the chick, the compensatory growth response to negative lenses seen at 500 lux is absent at 15000 lux (Ashby and Schaeffel, 2010). This is thought to be related to increased retinal dopamine levels in response to light exposure (Besharse and Iuvone, 1992), which is known to have an inhibitory effect on FDM (Stone, Lin et al. 1989). Extension of this idea has led to investigations with human children that suggest that outdoor light exposure may have a protective effect against myopia in children (Rose, Morgan et al. 2008).

2.6.4. Accommodation

In theory, a sufficiently flexible positive and negative accommodation system could negate the need for an emmetropisation system. Both accommodation and emmetropisation require a blur detection system, but how the eye differentiates short term blur that can be corrected by modifying accommodation, compared to blur caused by unbalanced ocular growth is not yet understood (Charman 2011). Several studies have shown that emmetropisation can function without influence from accommodation (Schaeffel, Troilo et al. 1990; McBrien, Moghaddam et al. 1993). While these studies eliminated functional accommodation, the biological scaffolding used to detect the optical defocus may still be functional, and shared with the emmetropisation mechanism (Troilo, Quinn et al 1997).

Evidence for two independent inputs to accommodation and emmetropisation, at least in primates, derives from the location of input for each. Accommodation appears largely driven by foveal signalling (Neil Charman and Radhakrishnan 2010; Graef and Schaeffel 2012), whereas the peripheral retina is locally responsive to form deprivation myopia (Smith III, Huang et al. 2009). There is also evidence that in the absence of a foveal signal, the peripheral retina alone can drive emmetropisation (Smith III, Ramamirtham et al. 2007).
2.7. Chromatic aberration as an input to emmetropisation

To this point, emmetropisation has been considered in terms of a single focal point or plane. However light rarely comes to a precise focus: instead, a complex point spread function is formed near the retina, with best spatial resolution achieved at the centre of mass of the three dimensional locus (van Meeteren 1974). In polychromatic light, one aberration responsible for creating a point spread function is chromatic aberration.

Chromatic aberration describes the splitting of polychromatic light into its chromatic components. Spread along the optical axis, it is called longitudinal (occasionally axial) chromatic aberration (LCA), and when along the oblique plane, transverse chromatic aberration (TCA) (see Figure 2.1). Both effectively degrade the image by creating multiple overlapping chromatically distinct images (He, Wang et al. 2013).

![Figure 2.1](image.png)

Figure 2.1 - Longitudinal chromatic aberration (a) creates a coloured point spread function along the visual axis, with blue light focusing anterior to red. Transverse chromatic aberration (b) deviates light perpendicular to the visual axis, with blue deviating more than red light.

2.7.1. Transverse chromatic aberration (TCA)

Any refractive lens, whether it be concave or convex, causes a lateral shift (prismatic deviation) for all non-paraxial rays, and this effect is greatest for shorter wavelengths. This is seen as coloured fringing around high contrast edges, such as text (Yang, Tai et al. 2011). As TCA increases with vitreous chamber elongation, it could be used as an input for emmetropisation.
However, there are several reasons why TCA is unlikely to play a significant role in emmetropisation when compared to LCA:

- The amount of aberration created by TCA is much smaller than LCA. Modelling the focal spread created by TCA on the retina estimates it at 1/8 the diameter of LCA (He, Wang et al. 2013). This makes it less detectable, especially when low order aberrations like refractive error are present.
- TCA increases the lateral separation between red and blue light as the distance from the optical components increases: there is no distance where it is zero. This means TCA is present in both an emmetropic and ametropic eye. For it to be used as a detector of emmetropia, an arbitrary amount of TCA, rather than an ideal amount (i.e. zero), would need to be targeted.
- The amount of TCA increases with increasing distance of the entrance light ray from the pupil centre (Thibos, Bradley et al. 1991). Therefore axial length is not the only factor affecting the amount of TCA, and the retina would need to determine the origin of the light through the pupil in order to differentiate the cause of TCA detected at the retina.
- In the human eye TCA varies significantly between subjects and even between eyes of the same subject (Marcos, Burns et al. 1999). This variance complicates both direction and magnitude detection, and would also require a method of self-calibration.

2.7.2. Longitudinal chromatic aberration (LCA)

LCA is a polychromatic optical aberration that results from different wavelengths of light being refracted by different amounts. The dissimilarity in refraction is due to each frequency of light interacting differently with the particles of the medium it is passing through, manifesting as variances in the refractive index (Smith and Atchison 1997). Light from a white point source, instead of focusing at a single point, creates coloured focal loci parallel with the optical axis. For most materials, LCA is positive, which means shorter wavelengths are refracted more than longer wavelengths (Rucker and Wallman 2009). Therefore, for the following reasons, LCA could provide a potential source for detecting the position of the focal plane relative to the retina:

- The impact of chromatic aberration on image quality is minimised when the eye is focused at the midpoint of an animal’s visual spectral range (Ravikumar, Thibos et al. 2008). In humans, whose spectral range extends from 380 to 700nm, optimal focus is
near 555nm (Flitcroft 1990). This gives a visual advantage to an eye focused at the centre of LCA.

- Due to the inverse exponential relationship between refractive power and radius of curvature of optical lenses, proportional errors in small eyes induce higher amounts of refractive error compared with larger eyes (Mutti, Mitchell et al. 2005). As LCA is manifest as a percentage of lens power, the absolute magnitude of LCA decreases as the eye increases in size (Wang, Candy et al. 2008). Expressed in terms of dioptres at the retina, in an infant eye, LCA can mask a relatively wide range of focal errors. As the eye grows (decreasing in optical power) and begins to emmetropise, LCA reduces in dioptic range (therefore increasing in sensitivity), which provides an inherent fine-tuning capability.

- As an image becomes myopically defocused, long wavelength (red) components of the retinal image have higher contrast than short wavelength (blue) components. The opposite is true for hyperopic defocus (Rucker and Wallman 2012). Such differences in chromatic contrast could provide a signal as to the direction of optical defocus at the retina (Marimount and Wandell 1994); they are balanced when the retina is at the centre of the LCA distribution.

- For an emmetropisation feedback loop to function on the basis of LCA, the retina would need a mechanism to compare contrast from two disparate points on the spectral sensitivity curve. In vertebrates, multiple opsin retinas are common (Khokhlova 2012), which could provide input to such a system.

2.7.3. Presence of LCA

For LCA to be used as a guide for emmetropisation, the aberration must be present at the retina (i.e. left uncorrected by the optical components of the eye). Significant chromatic aberration is reported in a variety of vertebrate eyes, including rock bass, frog, chicken, rat, cat, pig, and cow (Mandelman and Sivak 1983). Chromatic aberration is also present in the human eye, and was recognised as far back as Newton in his visionary treatise, Opticks (Newton 1730). Modern experiments in adult humans find approximately 2 dioptres difference in focal power for red and blue light, with little variation (reviewed in (Seidemann and Schaeffel 2002)). This translates to a longitudinal focal difference of approximately 720µm at the retina (He, Wang et al. 2013),
making LCA the single largest aberration in the polychromatic point spread function in the human eye (van Meeteren 1974).

### 2.7.4. Biological correction of chromatic aberration

Biological mechanisms which partially correct for LCA by using multifocal lenses have been reported in many species of fish (Gagnon, Söderberg et al. 2012), and some terrestrial vertebrates (Kroger, Campbell et al. 1999). For a polychromatic light source, a series of concentric zones in the crystalline lens provides an in-focus plane for wavelengths corresponding with peak cone sensitivities (Kroger, Campbell et al. 1999). The simultaneous viewing of the out of focus colours has a negative impact on maximum visual acuity. However, computer modelling suggests the trade-off may be beneficial overall (Gagnon, Söderberg et al. 2012). Such a system may have evolved as far back as the Cambrian (Gustafsson, Collin et al. 2008), as it is common amongst marine vertebrates (Malkki and Kröger 2005; Gustafsson, Ekström et al. 2012), and is also present in some extant land species (Lind, Kelber et al. 2008). The lack of ubiquity of such LCA correction might suggest that, at least in some species, it may be beneficial to retain LCA, or too detrimental to visual acuity to reduce it with a multifocal lens. In chicks, after reducing LCA with spectral filters, recovery from lens induced defocus can still occur (Rohrer, Schaeffel et al. 1992), as can recovery from lid suture induced myopia under monochromatic light (Wildsoet, Howland et al. 1993). While LCA does not appear to be an essential requirement for emmetropisation, when present, it may allow a more refined response to lens induced defocus.

### 2.8. Investigations on the role of wavelength of light in emmetropisation

There are two types of studies that examine the effect of wavelength on emmetropisation: those that shift the focal plane by exploiting inherent LCA, and those that investigate the effect of chromaticity.

#### 2.8.1. LCA as a method for shifting the focal plane

Within limits, the vertebrate eye appears to be able to emmetropise in a wavelength restricted light environment. Chicks raised in red light develop relatively myopic eyes when compared to
chicks raised in blue light, and the refractive error can be reversed by crossing over the lighting environment (Seidemann and Schaeffel 2002). However, while the chick visual spectrum extends into the UV (Wortel, Rugenbrink et al. 1987), UV sensitive photoreceptors are either not involved or of too low spatial resolution to be used for emmetropisation signalling (Rohrer, Schaeffel et al. 1992).

Guinea pigs raised under either short (430nm) or medium (530nm) wavelength LED lighting demonstrate a higher than expected response to the different focal planes, each selected to nominally stimulate different (L/M) cone pathways. After 12 weeks of exposure, the short wavelength group developed relative hyperopia of almost three times the estimated LCA (Liu, Qian et al. 2011). The difference in refraction was axial rather than refractive; the short wavelength group had a reduced vitreous chamber growth rate compared with the medium wavelength group. When the lighting groups were reversed at ten weeks, the guinea pig eye rapidly initiated a commensurate response, suggesting that the emmetropisation system can respond in both directions to chromatic focal planes. Both in response to the initial manipulation, and the crossover of wavelengths, the response of the eye exceeded the expected effect if LCA focal planes were the sole input. The authors suggested that in addition to the focal plane adjustment, the growth response may be driven by relative cone input (Qian, Dai et al. 2013). Others have also suggested that a balance of chromatically distinct pathways may be required to initiate a response when the eye is near emmetropia (Rucker 2013). Therefore, the overcompensation in refractive change seen in the guinea pig may be a result of the narrowband light affecting the relative input of the two cone pathways, preventing a comparison of the cone pathways that would otherwise occur in broad spectrum light.

Fish provide a useful model for studying emmetropisation. Unlike most terrestrial vertebrates, fish continue to grow into adulthood, presumably increasing the period over which emmetropisation is required. In the ocean, water acts as a blue filter, absorbing long wavelengths with increasing depth (Warrant and Locket 2004). This restricts the visual spectrum for aquatic animals, potentially reducing the value of LCA as an input for emmetropisation. Interestingly, LCA in a species of snapper (Lutjanus griseus) and striped burrfish (Chilomycterus schoepfi) is as high as 10 dioptres (Sivak and Bobier 1978). Optical modelling of the Rainbow Trout (Oncorhynchus mykiss) eye reveals similarly high uncorrected LCA of approximately 4% of the total lens power between 450-700nm (Jagger and Sands 1996). However, LCA is not
universally high in fish (summarised by Kröger and Campbell (Kröger and Campbell 1996), Table 4). Some fish species have evolved mechanisms to decrease the effect of LCA, such as a yellow pigmented cornea which can act as a spectral filter (Walls and Judd 1933).

Testing the effect of moving the plane of focus in a marine vertebrate was first investigated in a freshwater trichromatic African cichlid (*Haplochromis burtoni*) (Kröger and Fernald 1994). Under monochromatic LED illumination, selected to complement the peak sensitivity of each cone type (455, 523, 562nm), no difference in refraction or ocular dimensions was seen between each group, or a broadband white light group. This was possibly due to the poor sensitivity of the anatomical technique used to make measurements. However, later work showed this species to have lower than expected LCA (Kröger and Campbell 1996), which would have tended to minimise differences between groups.

Blue Acura (*Aequidens pulcher*) is another trichromatic cichlid which has been tested for an emmetropisation response by moving the chromatic focal planes. When raised under either red (623nm), green (534nm), blue (485m), or white light for six months, differences in naso-temporal eye size (correlating with areas of highest acuity) were seen between groups. Eyes from fish in the blue and green tanks were significantly smaller than eyes from the red tanks, though not significantly different from each other or the white control. This may be due to short-wave light filtering effects of water. When the fish previously housed in the red tank were later put into a white light environment, their relative eye sizes returned to the white light value within five weeks. The overall trend supported the expected result if the eye was emmetropising to the focal plane provided by the chromatic filters (Kröger and Wagner 1996).

### 2.8.2. LCA as a direct input for emmetropisation

Whether chromatic aberration is detected and used by the human emmetropisation system is poorly understood. However, several studies have investigated the role of LCA on accommodation control, which requires similar knowledge of the direction and magnitude of defocus.

LCA appears to be used as a guide for accommodation, with some individuals failing to respond to an accommodative stimulus under monochromatic lighting, while others demonstrate no change compared to polychromatic light (Fincham 1951). More recent studies also disagree
regarding the importance of LCA in the accommodative response (Lovasik and Kergoat 1988; Kruger, Nowbotsing et al. 1995), although most agree it is not essential, but can be utilised when present (Kruger, Mathews et al. 1993). Accommodation appears to target the middle of the focal spread created by LCA; this remains true when restricting wavelengths at either end of the visual spectrum (Kröger and Binder 2000). Humans, which lack blue cones at the fovea (Graef and Schaeffel 2012), demonstrate an unusually high accommodative response in blue light (Seidemann and Schaeffel 2002), termed OAB, or over-accommodation to blue. OAB is theorised to be due to lack of input from the red/green cone pathway, which draws similarities to the overcorrection apparent when isolating cone pathways in guinea pig emmetropisation (see 2.8.1). This provides evidence that colour information can be used to assist optimal focus during accommodation, and thus may potentially be used as an input for emmetropisation.

In a similar fashion to accommodative input, it could be that LCA is not essential for emmetropisation, but can be used as a guide when available. To determine whether the eye is capable of detecting LCA independently of the change in focal plane, the effect of LCA on the retina can be simulated in the environment by modifying the relative contrast in the red and blue; higher contrast in the red simulates a myopic focal plane, and higher blue contrast simulates a hyperopic focal plane (Rucker 2013). Exposing chicks to such an environment simulating 1.50D of LCA, chicks exposed to the simulated hyperopic defocus have thinner choroids, and less glycosaminoglycan (GAG) synthesis than those exposed to the simulated myopic defocus (Rucker and Wallman 2009). Their responses mirror the changes seen with defocussing lenses (Nickla, Wildsoet et al. 1997). This suggests that LCA does more than provide a range of focal planes, but that multiple chromatic planes of focus can be detected and compared in the chick visual system, and can initiate the early stages of compensatory eye growth. The details of this comparator are not known, but modelling of the human trichromatic retina shows that the colour opponent channels are capable of differential responses (Flitcroft 1990). If a cone contrast comparison is used as an input to emmetropisation, there are several apparent hurdles which may complicate the process. Aberrations vary considerably with pupil size (van Meeteren 1974), and large variations exist in long and medium cone retinal mosaics (Hofer, Carroll et al. 2005), which would complicate the input to the system.
2.9. Emmetropisation in cephalopods

There are no published reports of emmetropisation being investigated in invertebrates. The last common ancestor between the vertebrate and invertebrate clades existed prior to the Cambrian period (545-490 million years ago (MYA)), likely before the evolution of an optical eye (Lamb, Collin et al. 2007; Parker 2011). Camera-type eyes have been reported in a range of invertebrate phyla (e.g. cephalopods, gastropods, bivalves, crustaceans and annelids (Land 2012)), many of which might benefit from emmetropisation. A discussion of how the invertebrate camera type eye differs from the vertebrate form, and the potential methods for cephalopod emmetropisation are discussed in the following chapter.
Chapter 3. Cephalopods

3.1. Introduction

Cephalopoda (Greek: *kephalópoda*, “head-foot”) is a class of animals in the Mollusca phylum, which undisputedly contains the most intelligent invertebrates (Hanlon, Conroy et al. 2008; Finn, Tregenza et al. 2009; Andrews, Darmaillacq et al. 2013). Octopus in particular are considered highly intelligent, rivalling most land vertebrates by demonstrating attributes such as individual food preferences (Sinn, Perrin et al. 2001; Anderson, Wood et al. 2008), tool use (Finn, Tregenza et al. 2009), and personality (Sinn, Perrin et al. 2001; Prank, Wilson et al. 2010). However, care must be taken when assigning vertebrate attributes to cephalopods, as their last common ancestor existed prior to the Cambrian Explosion (Kröger, Servais et al. 2009) (see Section 3.5).

Cephalopoda includes four major marine groups; octopus, squid, nautilus, and cuttlefish. New Zealand has many coastal species of octopus and squid (O’Shea 1999; Gordon, Beaumont et al. 2010), which makes capture for study in the laboratory possible without deep sea voyages. Two different cephalopod species were used in this study: *Sepioteuthis australis*, the Southern Calamari Squid, and *Octopus tetricus*, the Gloomy Octopus.

3.2. Cephalopod anatomy

The basic body layout of a cephalopod consists of a soft outer shell, and a large muscular ‘foot’. In most cephalopods, the only extant exception being the Nautilus, the external shell common throughout Mollusca is no longer present. However, a vestigial component of the former shell remains inside the octopus mantle (Doubleday, White et al. 2011). The foot has been repurposed as arms and tentacles, and a funnel extends from the mantle through which water can be jetted through contraction of the ancestral shell manipulation muscles (Hanlon 1996). The mantle is a soft tissue performing the role of the shell, and includes an internal cavity which contains all the digestive, respiratory and reproductive organs. The circulatory system has three hearts, one central systemic heart, and two branchial hearts, one over each set of gills (v. Boletzky 1987). These hearts pump blood which appears blue because of copper based haemocyanin, rather than iron based haemoglobin present in vertebrates, as the oxygen carrying molecule (Melzner, Mark et al. 2007). The anterior end of the cephalopod has eight
arms lined with suckers, circularly organised around a central beak and radula, the invertebrate equivalent of a mouth, teeth, and tongue (Hanlon 1996). Squid, in addition to the eight arms, have two laterally opposed tentacles which can be rapidly projected to entrap prey (Kier and Schachat 2008).

3.3. Sepioteuthis australis

*Sepioteuthis australis* (Quoy and Gaimard, 1832, common name Southern Calamari or Southern Reef Squid, Figure 3.1) is a coastal squid found in shallow waters around Eastern Australia and Northern New Zealand (Triantafillos 2004). They are visually distinguished by their rounded, diamond shaped fins that are attached to the sides of their mantle (Norman and Reid 2000).

![Figure 3.1 - 60 day old Sepioteuthis australis. Visible are the two longer tentacles (+), prominent eyes (**) including lens and cornea), optic lobes (#) and several other internal organs. The beak, sitting inside the cone made by the arms, is visible between the eyes (^). Scale bar is 10mm.](image)

*Sepioteuthis australis* is fished commercially, and demand for calamari (squid, when prepared as food) has increased pressure on squid populations (Steer, Fowler et al. 2005). As such, most *S. australis* research is conducted with ecological motivations, in order to provide better understanding of their lifecycle and behaviours to ensure long term sustainability (Boyle 2004).
3.3.1. Squid anatomical terms of location

The squid anatomical terms of location are similar to that of fish, yet slightly harder to define as squid swim in both directions. They adopt an "arm forward" direction when hunting, yet can swim more quickly "mantle forward", by jetting water through their funnel. The ventral side of the dorsal-ventral axis is identified by the tip of the funnel exiting the mantle hood, which can be moved to either side of an internal septum. If the squid is inverted it will quickly try to right itself to dorsal side up. The dextro-sinister (left-right) axis can be determined from a top-down view, viewing the dorsal side with the arms up (Figure 3.2). The beak (mouth) is towards the anterior end of the animal (between the arms), defining the longitudinal anterior-posterior axis.

Figure 3.2 - Anatomical terms of location used in this thesis for a Sepioteuthis australis right eye. The arms and tentacles are on the right of this top-down dorsal view, the left eye is out of focus near the top of the image. The spherical lens extends through the pupil, with the iris descending from the superior of the eye over the lens surface. An attempt has been made to use terms common in vertebrate eye studies to improve interpretation of results.

The anatomical terms of reference for the eye can be defined in a similar fashion to a vertebrate eye. The dorsal-ventral axis is continued from the overall anatomy, but is instead defined as superior-inferior; the iris closes from superior to inferior. The anterior-posterior axis is 90 degrees rotated to that of the body layout – and is synonymous with the optical axis (lens-retina direction), and used in preference to distal/proximal to provide commonality with equivalent
vertebrate studies. Defining the left–right axis as such could lead to confusion as to which eye is being referenced, so the terms medial for towards the arms, and lateral, for towards the mantle will be used instead. This is similar to the vertebrate definition, where the eyes converge together by rotating medially.

3.3.2. Rearing *Sepioteuthis australis*

Squid have been of interest since as experimental subjects since the seminal work on action potential conduction in the squid giant axon in wild caught specimens (Hodgkin and Huxley 1952). Since then, there has been interest in squid as an experimental species, to discover what else can be learnt from their convergently evolved organ systems (Gilbert DL, Adelman WJ Jr. et al. 1990). The research interest extends beyond the eye, and includes the nervous (Hochner 2013) and circulatory systems (Yoshida, Shigeno et al. 2010), as well as interesting questions such as the evolution of consciousness (Mather 2008), memory (Brown and Piscopo 2013), and personality (Prank, Wilson et al. 2010). Unfortunately, working with squid is difficult, as the squid either have to be captured at sea, which can risk damaging them (Sweeney, Haddock et al. 2007), or by maintaining squid in a laboratory.

Unfortunately, keeping squid in a laboratory is challenging. The main difficulties include procuring a sufficient amount of appropriate food, and the need for a relatively complex and large aquatic setup to maintain high water quality and offer squid enough space to prevent abrasions and secondary bacterial infections (Yang, Hanlon et al. 1983; Yang, Hanlon et al. 1989; Hunt, Steer et al. 2011). After hatching, the yolk that has been feeding the squid inside the egg (Figure 3.3) is almost depleted, and hunting must be successful within a few days or else weight and the ability to successfully hunt, diminishes towards a point of no return after four days (Vidal, DiMarco et al. 2006).
Figure 3.3 – Deceased *Sepioteuthis australis* and its yolk at developmental stage 27 (of 30). In this case the yolk (‘’) is separated from the squid. The visible tip of the yolk (>) was previously inside the digestive tract; the smaller ball (+) held within the arms. During the last 3 developmental stages, the squid absorbs the majority of the remaining yolk, causing the squid to significantly increase in size. The squid hatches with residual yolk retained in the digestive tract which can sustain the squid for a short period outside the egg. Scale bar in bottom right is 1mm.

*Sepioteuthis sp.* were first successfully hatched and kept for 45 days in an artificial (but open to the sea) environment in 1963 (Choe and Ohshima 1963). These were *Sepioteuthis lessoniana*, unusual amongst *Sepioteuthis sp.* in that they have very large eggs at hatching, with a mantle length of approximately 5-7mm (Ikeda, Wada et al. 1999). This is much larger than *S. australis* hatchlings, and makes the task of capturing food for the squid easier. Of the smaller egg laying squid, the first to be raised was *Loligo opalescens* (hatching mantle length 2.8mm), which was maintained to a maximum of 100 days in 1976 (Hurley 1976). Hurley’s aquarium required frequent and large water exchanges in order to preserve water quality (i.e. not recirculating water), and the squid were undersized, with a maximum mantle length of 13mm at 80 days (Yang, Hanlon et al. 1983).
Success at rearing *L. opalescens* in a fully closed system was achieved by Hanlon in 1979 (Hanlon, Hixon et al. 1979), who maintained squid for 79 days, until they reached a mantle length of 17.3mm. This was extended four years later by keeping *L. opalescens* to a maximum of 233 days (Yang, Hanlon et al. 1983). Yang et al. later completed a full life cycle, describing it, along with *S. lessoniana* in 1989 (Yang, Hanlon et al. 1989). *S. lessoniana* has now been successfully cultured in captivity over many squid life cycles (Lee, Turk et al. 1994; Walsh, Turk et al. 2002). More recently, several others have described success at hatching squid from eggs (Staaf, Camarillo-Coop et al. 2008; Bozzano, Pankhurst et al. 2009), holding squid captive for a number of days (up to 102 days (Pecl 2004)), and getting squid to lay eggs in a tank (Staaf, Camarillo-Coop et al. 2008).

*Sepioteuthis australis*, the species used in this study, has only recently been successfully raised from hatchlings in a laboratory (Hunt, Steer et al. 2011), although the squid only survived for 40 days. In this study we have successfully raised *S. australis*, from -20 days (pre-hatching) to over 6 months old. Full details of the aquaria setup and protocols used are provided in Appendix 1.

**3.4. Octopus tetricus**

*Octopus tetricus* (Gould, 1852, common name Gloomy Octopus, Figure 3.4) is one of 42 species of octopus found in New Zealand waters (O’Shea and Wassilieff 2012). Octopuses can adopt a wide variety of physical appearances (colour, shape, and texture), and this can make classification of species difficult (Acosta-Jofré, Sahade et al. 2012). Currently, *O. tetricus* is best described as a group of virtually indistinguishable species. It has recently been claimed that the octopus used in this study should be regarded as a separate species (*O. gibbsi*), but at the time of writing this claim had not been accepted (Norman and Hochberg 2005; Bouchet 2013).

*O. tetricus* is a benthic species, which lives in shallow water around the East coast of Australia and the Northern coasts of New Zealand (Anderson 1997). Like *Sepioteuthis sp.*, it is also fished commercially, although *O. tetricus* makes up only a small proportion of total octopus catches (NSW Wild Fisheries Research Program 2010).

As only adult octopus were used in this study, the captive maintenance was easier than for squid, although the tanks had to be specifically designed (see Appendix 1). Keeping octopus in
captivity has been well documented (Forsythe and Hanlon 1980; Brown, Piscopo et al. 2006; Rodriguez, Carrasco et al. 2006), and unlike squid, is routinely achieved in public aquariums, and by keen home aquarists.

Figure 3.4 - Our smallest Octopus tetricus, Octavia, finishing her lunch of green lipped mussels. A terracotta pot, which served as her den, can be seen in the background. The octopuses quickly adapted to humans, showed no fear when people moved around the tanks, and could be hand fed, trained to open jars, and interact in a manner similar to playing games.

3.5. Phylogenetic separation of cephalopods and humans

Cephalopods belong to the invertebrate phylum Mollusca, whereas humans are Mammalian vertebrates (Figure 3.5). Both cephalopods and vertebrates have bilateral symmetry, and are comprised of three cell layers (triploblastic); however, their evolutionary history diverges shortly after the emergence of these features. During early development of an embryo, an indentation (blastopore) forms, which deepens and becomes one end of the gastrointestinal tract (Hejnol and Martindale 2008). Cephalopods, like insects, are protostomes, which means the blastopore becomes the mouth end of the gastrointestinal tract, as opposed to humans, and all other vertebrates, which are deuterostomes, where the blastopore becomes the anus. This split is well
preserved in the embryology of all extant triploblastic animals, so therefore this can allude to the phylogenetic distance between two species (Lynch 1999).

Figure 3.5 – The evolutionary relationship of extant phylum which contain species with image forming eyes. Chordata, to which humans and all existing animal models of emmetropisation belong to, evolved from the Deuterostome group of animals during the early or pre-Cambrian. The five other phyla which possess image forming eyes are all Protostomes. These phyla, including the largest phylum, Anthropods, is dominated by compound type eyes. Mollusca are a notable exception, as they contain species with camera type eyes comparable to those found in Chordata. The last common ancestor between Deuterostomes and Protostomes existed prior to the diversity of phenotypes (including eyes with lenses) that occurred during the Cambrian explosion, 540 million years ago (MYA). Camera-type eyes are present in some Cnidarian jellyfish, who contain just two cell layers, placing their last common ancestor with humans prior to triploblasty, or further than 640 million years ago.

Determining when in history this split occurred is difficult, due to the poor fossilisation of soft-bodied animals (Erwin and Davidson 2002) and significant geological changes that have occurred since. However, comparison of essential protein-coding gene sequences (a technique called a ‘protein clock’ (Doolittle, Feng et al. 1996)) in protostomes and deuterostomes places the divergence up to 850 million years ago (Feng, Cho et al. 1997; Gu 1998). Conversely, when comparing mitochondrial DNA sequences (using a ‘molecular clock’ (Zuckerkandl and Pauling 1962)), the split may be as recent as 600 million years ago. Either technique places the last common ancestor of humans and cephalopods earlier than the Cambrian explosion, which
occurred approximately 540 million years ago (Kröger, Servais et al. 2009; Ginsburg and Jablonka 2010). Therefore, the phylogenetic distance, or the distinct evolutionary time that two species have had to independently evolve, is greater than one billion years. Considering a pessimistic estimate that an eye may evolve from a light sensitive pit to an image forming, camera-type eye in as few as 364 000 years (Nilsson and Pelger 1994), the potential diversity between the extant cephalopod and human eyes can not be understated. A more distant phylum from humans would be the Cnidarians, most of which have only two cell layers (Boero, Bouillon et al. 2005; Park, Hwang et al. 2012). While some Cnidarian species have image forming eyes (Nilsson, Gislén et al. 2005), they are well out of focus (hyperopic) and lack sufficient neurons to process the visual information (Wehner 2005), so the lens in the eye is more likely used for light gathering and gross orientation, rather than its ability to form a clear image (O’Connor, Nilsson et al. 2010; Petie, Garm et al. 2013). Like the cephalopod eye, the Cnidarian image-forming eye has evolved independently of the vertebrate eye (Kozmik, Ruzickova et al. 2008).

3.6. Convergent evolution of camera-type eyes

Octopus, squid and humans all have camera-type eyes (Figure 3.6). Camera-type eyes have an iris, which can adjust the amount of light entering the eye, and a lens which can adjust the power of the eye in order to provide clear focus at a range of distances (Land 2002). Despite these optical similarities between cephalopod and human eyes, both designs developed along distinct evolutionary pathways as their last common ancestor, prior to the rapid development during the Cambrian explosion, lacked an optical eye (Arendt 2003; Nilsson 2013). The similar optical design creates a common need for alignment of the focal plane and retina to achieve maximum visual performance. While the evolution of emmetropisation has not previously been investigated, once images began to be formed in eyes, pairing of axial eye length with the focal length of the optical components becomes evolutionary advantageous.
3.7. Commonalities and contrasts between cephalopod and vertebrate eyes

Sensitivity to light extends further back in evolutionary history than the last common ancestor of cephalopods and vertebrates (Feuda, Hamilton et al. 2012). Therefore some aspects of vision are shared between the two groups, whereas others have evolved independently more recently. Any development of the eye or visual system prior to the Cambrian may be shared between Mammals and Molluscs, but anything more recent has independently evolved.

3.7.1. Opsins

Vision relies on the ability of a biological system to convert energy from photons of light into a biological response. This is achieved with a seven transmembrane G-protein coupled receptor with a bound chromophore molecule, collectively called an opsin (Feuda, Hamilton et al. 2012). Opsins undergo a conformational change in response to photon capture, which initiates a biological cascade (Peirson, Haiford et al. 2009).

There are two types of opsin molecules with the Animalia kingdom, which share significant structural and functional similarities but whose primary amino acid structure suggests independent evolution (Spudich, Yang et al. 2000). The evolutionary history places the emergence of both proteins prior to the last common ancestor of cephalopods and vertebrates (Santillo, Orlando et al. 2006). Type 1 opsin, also called bacteriorhodopsin, is present in prokaryotes, and some lower order eukaryotic bacteria. Type 2 opsin is exclusively and ubiquitously found amongst all higher order eukaryotes (Ballottari, Girardon et al. 2012).
With octopus, squid, and humans existing well down the eukaryote branch (with Type 2 opsins), the differences between the two classes of opsin are not particularly relevant to this study, and will not be discussed further. An interested reader is referred to the comprehensive review by Spudich et al (Spudich, Yang et al. 2000). Opsin sensitivity to different wavelengths of light however, is important to the design of the experiments in this thesis, and is discussed in greater detail in the experimental design (see Chapter 5.4).

3.7.2. Photoreceptors

In the extant animal kingdom, there are two classes of photoreceptors, ciliary and rhabdomeric (see Figure 3.7). With a cursory glance, invertebrate eyes, including those of cephalopods, contain rhabdomeric photoreceptors and vertebrate, including human eyes, ciliary photoreceptors. Unsurprisingly, it was traditionally believed that these photoreceptors convergently evolved after the last vertebrate/invertebrate ancestor (Eakin 1965). However, recent evidence has revealed several animals with the ‘wrong’ photoreceptors (Arendt and Wittbrodt 2001; Arendt, Tessmar-Raible et al. 2004; Passamanec, Furchheim et al. 2011).

Additionally, the recently discovered intrinsically photosensitive Retinal Ganglion Cells (ipRGC) (Foster and Hankins 2002) in vertebrates show many similarities to rhabdomeric photoreceptors (Graham, Wong et al. 2008), having melanopsin, an opsin protein homologous with the invertebrate form (Berson 2007; Graham, Wong et al. 2008) and a rhabdomeric phototransduction cascade (Graham, Wong et al. 2008). Moreover, ciliary photoreceptors with vertebrate opsins have been discovered in the brain of the invertebrate Platynereis (Arendt, Tessmar-Raible et al. 2004). Additionally, some cubomedusan jellyfish (phyla: cnidarians, a diplobastic animal) have both ciliary and rhabdomeric photoreceptor types (Arendt and Wittbrodt 2001; Sirakov, Zarrella et al. 2009).
Vertebrates possess ciliary photoreceptors (a), with photopigment distributed along a modified cilium (\textasteriskcentered) that polarises to light. Cephalopods have rhabdomeric photoreceptors (b), where the photopigment is distributed along apical microvilli (+). In contrast to ciliary cells, rhabdomeric photoreceptors depolarise to light and dominate the invertebrate world. The direction of light is from the bottom of the image, showing how the ciliary photoreceptors are orientated away from the direction of light – an embryonic peculiarity. The anthropomorphically named inner (IS) and outer segments (OS) of the photoreceptor cells are labelled.

The finding that both photoreceptor types are present in vertebrates and invertebrates places the appearance of photoreceptor cells prior to the protosome and deuterosome split (Lamb 2009). This lends support to a common ancestor possessing both types of photoreceptors, with the preference for one or the other becoming the primary light sensing cell occurring later (Arendt and Wittbrodt 2001; Arendt 2003; Erclik, Hartenstein et al. 2009).

3.7.2.1. Phototransduction

Phototransduction describes the process in which energy from light is translated into a biological signal; the exact series of biochemical reactions is termed the photocascade, and this cascade varies significantly between cephalopods and vertebrates.

The vertebrate photocascade is unique in that photon capture causes hyperpolarisation of the photoreceptor cell (Takimoto, Kusakabe et al. 2007; Kusakabe, Takimoto et al. 2009). In the
absence of light, open sodium channels in the photoreceptor outer segment permit a dark current, which maintains the resting membrane potential at approximately -40mV. The evolution of this cycle is unclear, but could be due to replication of previously non-functional, or non-visual genes (Albalat 2012).

The invertebrate photocascade differs significantly, with photon capture resulting in depolarisation of the photoreceptor cell (Pepe 2001). The invertebrate photocascade closely mimics other neural activity, including ganglion cell activity in the vertebrate retina (Graham, Wong et al. 2008). This may suggest a common ancestor with both photoreceptor types, or that the invertebrate phototransduction cascade is a modification of an existing G-protein response (Nakagawa, Iwasa et al. 1999).

3.7.3. Retina

The vertebrate retina is a complex neural structure, and includes ciliary photoreceptors, bipolar cells, and ganglion cells (which exit the eye as the optic nerve). At the bipolar cell level, horizontal and amacrine cells modify signals from the photoreceptors and bipolar cells (Purnyn 2013). Vertebrate retinas have a high degree of neural convergence; a human retina, for example, contains approximately 85 million photoreceptor cells (Panda-Jonas, Jonas et al. 1994), yet the afferent optic nerve contains just one million fibres (Jonas, Muller-Bergh et al. 1990). Additionally, the photoreceptors output is divided into multiple simultaneous streams, which are processed by over 60 distinct cells types in the retina (Masland 2012). Therefore, the vertebrate retina is involved in significant processing of the visual signal, before the signals exits the eye (Purnyn 2013).

Also, the photoreceptors in the vertebrate retina, which are the most distal component of the retinal network, lie deeper in the retina than the more proximal ganglion cells, which project out of the eye. This means a photon has to traverse multiple layers of neurons, before being captured by the photoreceptor outer-segments (Figure 3.8). This unusual arrangement can be traced to the embryology of the eye, with the inversion occurring due to an invagination of an initial outpocket of neural tube (the optic vesicle) (Lamb, Collin et al. 2007).
Figure 3.8 - Comparison of a (a) cephalopod and (b) vertebrate camera-type eye. As the cephalopod cornea is neutralised in water, the lens provides almost all of the refractive power. Light entering the posterior chamber can be immediately absorbed by the forward pointing photoreceptors in the single layered cephalopod retina, which output via depolarisation to an external optic lobe. In the human eye, the lens only provides 1/3 of the refractive power, and is oblate when at rest. A photon in this eye must pass through multiple orders of neurons (and possibly a blood vessel layer) before getting absorbed by the rearward pointing rhabdomeric photoreceptors. These photoreceptors hyperpolarise in response to light, which gets modulated in the retina before exiting the eye as a depolarisation via the ganglion cell layer.

In comparison, the cephalopod retina is simple. It contain only photoreceptors, supporting glial cells, and the distal ends of efferent fibres (Young 1971). The photoreceptors are all rhabdomeric, arranged in repeated orthogonal patterns termed rhabdomeres, which permits discrimination of polarised light (Shashar and Cronin 1996). With infrequent exceptions (Oba and Kainuma 2009), the cephalopod retina contains only one photopigment, with a peak sensitivity of approximately 494nm (Muntz and Johnson 1978; Morris, Bowmaker et al. 1993). The cephalopod retina lacks a true anatomical fovea, but contains increasing photoreceptor density towards the posterior pole; a horizontal band in octopus, and a circular region in squid closely matching their respective pupil shape (Talbot and Marshall 2011). This suggests areas of improved visual acuity; the pole is also likely to have the lowest optical aberrations (Mass and Supin 2007). The axons from the photoreceptors converge along a horizontal equatorial axis, and intertwine before piercing the sclera in 10-15 bundles of fibres (Young 1962). The axons
project to the optic lobe, which houses all the post–processing cells in a remarkably similar fashion to the vertebrate retina, only extra-ocularly (Young 1962; Williamson, Ichikawa et al. 1993; Williamson and Chrachri 2004).

### 3.7.4. Anterior eye

The human eye contains two refractive components: the cornea, which provides almost 2/3 of the refractive power, and the remainder is provided by the crystalline lens. The cephalopod eye is underwater, so the optical power of the corneal surface, which is present in myopsid (coastal) squid and most costal octopus, is nullified by the almost equivalent refractive index of the surrounding water. Therefore, cephalopod eyes effectively have only one refractive component, the crystalline lens. This lens is proportionately larger, rounder, and more optically powerful and would be expected to induce more spherical aberration than the vertebrate counterpart. However, the refractive index of the cephalopod lens increases towards the nucleus of the lens which corrects the majority of the spherical aberration (Jagger and Sands 1999; Land 2012). Marine vertebrates, such as fish, also have a similar spherical lens design (Jagger and Sands 1996).

The origins of vertebrate and cephalopod lenses are very different. The vertebrate lens forms from an invagination of the embryonic surface ectoderm into the optic cup (Chow and Lang 2001). The cephalopod lens is composed of two portions, anterior and posterior, which are fused with a septum which may regulate ion transfer between the two portions (Jacob and Duncan 1981). Each portion has a physically distinct ectodermal origin; the posterior portion develops early, while the anterior portion continues to grow proportionally more quickly, making up 17% of the total lens diameter at birth, but over 35% in the adult (West, Sivak et al. 1995). While the evolution of the cephalopod lens is well understood (Sweeney, Des Marais et al. 2007; Piatigorsky 2008), any advantage that having two distinct portions may provide is not clear.

### 3.7.5. Genes

While an exact phenotype may be influenced by the environment (epigenetics), the gradual evolution of the eye over generations is determined by changes in DNA. Ideally, we would compare the DNA of an extant animal with a series of extinct species in order to date the
emergence of certain genes, but there are limits on the survivability of DNA over time. In the absence of intact primitive DNA, study of the homology of genes between extant clades can yield clues as to the evolution of such genes (Park, Hwang et al. 2012).

Gene analysis has shown remarkable similarities between invertebrate and vertebrate Pax-6 genes, with Drosophila possessing 94% sequence similarity to that of the human form (Quiring, Walldorf et al. 1994). Alteration of the expression of Pax-6 can result in fully developed ectopic eyes in Drosophila which can show a normal electrophysiological response, but lack appropriate afferent neural pathways (Gehring and Ikeo 1999). Infusion of the mouse Pax-6 gene into ectopic sites on Drosophila, which would normally encode a vertebrate camera-type eye, results in functioning compound eyes (Halder, Callaerts et al. 1995). The reverse - infusion of the invertebrate Pax-6 gene into a vertebrate - results in the development of the appropriate vertebrate eye (Chow, Altmann et al. 1999). The conservation of such genes strongly suggest a common genetic ancestry, and that Pax-6 may be the 'master control gene' behind eye development (Treisman 2004; Callaerts, Clements et al. 2006; Von Salvini-Plawen 2008).

The preservation of Pax-6 over millions of years of evolutionary time is likely due to genetic defects having extremely detrimental consequences for visual development and performance (and consequently reproductive success). In humans, heterozygous defects in Pax-6 lead to aniridia and a reduction in eye size (Bayrakli, Guney et al. 2009). In mice, homozygous defects cause a complete lack of eye development, as well as gross cranial and neurological defects such as lack of nasal development, which makes the first breath impossible (Hill, Favor et al. 1991; Dellovade, Pfaff et al. 1998). The Nautilus, the only member of Cephalopoda lacking a crystalline lens, has several mutations in the six3/6-like gene, which is required for lens formation in vertebrates, and therefore may have prevented the evolution of an image forming eye (Ogura, Yoshida et al. 2013).

Jellyfish (phyla: cnidarian) are largely (Boero, Bouillon et al. 2005)) diploblastic organisms which branched off our evolutionary roots prior to bilateria (Seipel and Schmid 2005). Jellyfish nervous systems are sparse and decentralised, yet despite this, there are examples of ocelli, pit eyes, and camera eyes in jellyfish (Kozmik, Ruzickova et al. 2008). A gene, Pax-B, has been identified (Kozmik, Daube et al. 2003), which shares some characteristics with Pax-6, leading some to speculate that Pax-B may be the evolutionary father of Pax-6, and that all bilateria began with
the same genetic encoding for eye development. As Gehring states, a finding of a Pax-6 like gene in a cnidarian would refute this theory, but so far none have been found (Gehring 2004; Gehring 2005).

3.8. Rationale for using cephalopods as a model for emmetropisation

A common approach to using animals to study human disease is to select a species that is phylogenetically similar to humans. This is based on the assumption that the more closely related an animal is to humans, the better model will translate to the human condition (Head 2013; Malafoglia, Bryant et al. 2013). Unfortunately, this raises the question of just how similar the animal is to humans, and therefore how accurate the model of disease may be (Zadnik and Mijtti 1995; Laurijssens, Aujard et al. 2013). This study approaches the problem of understanding emmetropisation differently. Instead of selecting an animal model that may have similarities with the human condition, a species was used which faces the same optical challenges, but shares almost no evolutionary ocular development. The vast majority of the eyes of invertebrates are of the compound type (Cronin and Porter 2010), however, simple type eyes are present in the invertebrate Mollusca phylum. While of similar design, the cephalopod and vertebrate eye have been simultaneously evolving since before the Cambrian Explosion. Despite this large phylogenetic distance, today they both possess an eye of similar function, and presumably share a common need for emmetropisation. The convergent evolution of the cephalopod camera-type eye appears to have created a simpler model for emmetropisation, and squid themselves have appealing characteristics which makes them an exciting model for emmetropisation.

3.8.1. Squid are visual predators

Squid are active predators, and need to hunt from an early age. As there is no parental guidance, and huge competition from neighbouring hatchlings, a juvenile squid must learn to hunt within a few days of hatching (Vidal, DiMarco et al. 2006). Supported by repeated observations made during the course of this study, squid hunting appears to be based predominantly on visual cues (Gilbert DL, Adelman WJ Jr. et al. 1990). While stalking or investigating new prey, the squid initiates a very quick rotation to orientate itself in the direction
of the prey, with its arms pointing forward in a cone. The eyes slightly converge medially so that they appear to look down the cone while it positions itself. After capture of large prey, the eyes can be seen to converge while the arms manipulate the food (Figure 3.9). Cephalopods visually observe their surroundings prior to hatching (Guibé, Poirel et al. 2012), and early observation can affect later prey preferences (Darmaillacq, Chichery et al. 2006). Communication between cephalopods is also likely visual (Mäthger, Denton et al. 2009; Mäthger, Shashar et al. 2009).

Figure 3.9 - Sepioteuthis australis having just captured a fish. The left eye can still be seen converging slightly as the fish is manipulated towards the beak at the base of the cone formed by the arms. A video demonstrating convergence can be seen at http://youtu.be/GFpq39oLB04.

In older squid (from about 10 days post-hatching), squid were seen positioning themselves behind their prey, which increased the successful capture rate. After the addition of a new prey type (especially the first fish), squid would slowly approach and observe before deciding whether to attack. Once familiar with the prey however, the attack would be very quick, and the squid would hover near the opening of the net waiting for the prey to emerge, having presumably recognised it while inside the net.
It is hypothesised that if the squid eye is not able to obtain a clear image shortly after birth, then the squid is less able to hunt, and thus would have a lower chance of surviving. The simultaneous spawning of hundreds of siblings ensures intense competition immediately from hatching; those with the best ability to detect prey will benefit at the expense of the rest. This early natural selection may help drive both optimal visual acuity at birth, and emmetropisation from day one.

### 3.8.2. Squid eyes grow over a much larger range than human eyes

Human eyes maintain emmetropia while they increase in size by almost 50%, from an eye length of approximately 17mm at birth, to about 25mm as a young adult. The *Sepioteuthis australis* in this study had a 0.5mm diameter eye cup at birth, which grew to 4.5mm in 60 days (see Figure 3.10 for a scale comparison), a growth of 900%. Some species, such as the giant squid, are born with a similarly small body and eye size, yet its eyes grow to almost 30cm as adults (Nilsson, Warrant et al. 2012). Squid also continue to grow for almost their whole lifespan (Arkhipkin and Roa-Ureta 2005).

![Figure 3.10 – Scale diagram of the relative eye size at birth (saturated) and sexual maturity (desaturated) for the ocular globe of *Homo sapiens* (green) and the hemispherical eyecup of *Sepioteuthis australis* (blue). While the human eye grows slightly from birth; the *Sepioteuthis* eye increases in size multiple times. The largest squid eye (from the colossal squid) has a diameter an order of magnitude larger than a mature human eye (not shown). Scale bar in top right is 10mm.](image)
As eyes increase in size, their refractive power decreases (Mutti, Mitchell et al. 2005). Therefore, compared to a human eye, which slightly increases in size over a small proportion of a lifespan, an effective emmetropisation mechanism in a squid eye would need to cover a greater range of refractive errors, and remain active over the entire life of the squid.

### 3.8.3. The cephalopod retina is much simpler

Part of the difficulty in understanding emmetropisation is that existing vertebrate models possess complex retinas: over 60 distinct neuron types have been identified in the mammalian retina (Masland 2012). Most vertebrates possess some ability to discriminate colour, which requires integration or comparison of multiple photoreceptor pathways (Jacobs 2012). As discussed in Chapter 2, emmetropisation in vertebrates appears to be largely local to the eye. What is not known, however, is whether this complexity contained in the retina is essential for emmetropisation.

The cephalopod retina is comparatively simpler, as it contains only a single order of neurons, the photoreceptors (see section 3.7.3). Additionally, most squid also only have a single opsin molecule, which reduces the potential signal convergence pathways.

This means if emmetropisation exists, either the signal, including the direction of defocus, is encoded in the output of the eye, and processed offsite, or the feedback loop is contained within the photoreceptor cells themselves. Either possibility offers exciting opportunities for further localisation, and may offer insight into the potential simplicity of emmetropisation in the vertebrate eye.

### 3.8.4. Cephalopod eyes are uncorrected for LCA

From an evolutionary view, visual acuity can be improved by reducing chromatic aberration within the eye’s optics (Ravikumar, Thibos et al. 2008). There are biological mechanisms that can correct for LCA (see Chapter 2.7.4); so why are they not universal? One possibility is that for LCA to be useful as a guide for emmetropisation, the aberration must be left sufficiently uncorrected.

While the cephalopod lens is well corrected for spherical aberration, longitudinal chromatic aberration (LCA) is generally poorly corrected. With a single opsin molecule, this may be
thought to be irrelevant, but in a similar fashion to spherical aberration, a non-monochromatic light source will create a focal spread of coloured images, causing a focal point spread function similar to that of monochromatic spherical aberration (Ravikumar, Thibos et al. 2008). In 1928, Heidermanns (discussed in (Jagger and Sands 1999)) described chromatic fringing when looking through an octopus lens. More recently Jagger and Sands (Jagger and Sands 1999) investigated LCA while developing a model for the octopus lens. Using 30nm bandwidth filters, they found approximately 4% difference in focal length between 450nm and 700nm.

One species of cephalopod has evolved a mechanism to minimise longitudinal chromatic aberration; the Japanese Firefly Squid (*Watasenia scintillans*), unique in that it possesses multiple chromophores (Seidou, Sugahara et al. 1990). Unlike other squid species, it controls for LCA by having a banked retina, with each layer containing photoreceptors with a different sensitivity, so that each layer receives an in focus image simultaneously (Kröger and Gislén 2004). This type of allowance for LCA at the retinal level (leaving the actual optical signal intact), would still allow LCA to be used as an emmetropisation input, but the mechanism may be more similar to a vertebrate mechanism, where comparisons between different chromophore input channels can be compared (Rucker 2013).
Chapter 4. Thesis Aims

Cephalopods present a unique opportunity to investigate emmetropisation from an evolutionary perspective. Their convergently evolved camera-type eyes would seem to require accurately controlled refractive development in a similar manner to that of vertebrates, including humans. However, both the optics and the retinal anatomy of cephalopod eyes are much simpler than those of vertebrate eyes. Experiments were conducted on this simpler cephalopod eye with the aim of obtaining a different perspective on emmetropisation, which may in turn provide insight into the function of the more complicated vertebrate system. However, cephalopods are not a common research animal, and no appropriate facilities existed within The University of Auckland prior to this thesis work.

**Aim 1:** To build and maintain a recycled salt-water aquarium in a sixth-floor animal facility, capable of keeping multiple cohorts of cephalopods alive for extended periods.

The establishment of two independent 1000 litre closed-system aquaria (one for squid, one for octopus) took considerable time, but eventually the balanced ecosystems reached a level of near autonomy. The details of how this aim was achieved are provided in Appendix 1.

**Aim 2:** To procure and house adult octopuses for several weeks, and to successfully procure, hatch and raise squid to sexual maturity in the recycled water aquarium.

Once the aquaria were established and stable, the next challenge was cephalopod husbandry. Raising and maintaining cephalopods in captivity is challenging, and is usually achieved in systems open to the ocean (see Chapter 3 for a discussion of previous attempts). During the course of this thesis work, three adult octopuses were kept for several weeks, and five independent cohorts of squid were successfully raised from egg-masses to adulthood. The
details of rearing and maintaining squid and octopus are provided in Appendix 1. All these animals were suitable for use in the subsequent experiments.

The major objectives included (a) determining whether squid possess active, visually guided emmetropisation, and (b) obtaining measurements of visual acuity in both squid and octopus.

**Aim 3**: To obtain repeated measures of ocular dimensions and refractions of squid eyes subjected to different spectral lighting conditions during development.

The design of the chromatic experiments and techniques used for measuring experimental outcomes are described in Chapter 5; the results of the chromatic experiments on squid eye growth are presented in Chapter 6. A discussion of the results is provided in Chapter 7.

**Aim 4**: To create a psychophysical method for measuring octopus visual acuity, and obtain an estimate of squid visual acuity by measuring their lens modulation transfer function (MTF).

The details of the novel methodology employed to measure octopus visual acuity can be found in Chapter 8, along with the details for determining the squid lens MTF. The results of both experiments are presented in Chapter 9, with a discussion in Chapter 10.

A concluding summary, including potential future areas of study, can be found in Chapter 11.
Chapter 5. Methods: Emmetropisation in Sepioteuthis australis

5.1. Introduction

To test whether Sepioteuthis australis possesses visually guided emmetropisation, three experiments were undertaken. The first experiment simultaneously raised two cohorts of squid in different spectral environments to determine whether there was a difference in eye growth. The second experiment examined whether the effect could be reversed by switching the squid between the different spectral environments. The third experiment extended this further by switching the squid multiple times between environments, earlier in the squid life cycle.

5.2. Experimental subjects

All experiment procedures were approved by the Animal Ethics Committee of The University of Auckland, and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Several squid egg masses were hand-collected in shallow water from the Hauraki Gulf, off Auckland’s east coast (approximate Latitude: 36.8S, Longitude: 174.8E) during the summers of 2010-2013. A total of 16 egg masses were collected, 9 of which hatched, and 5 of which survived the juvenile period and were successfully used for the experiments, including four serial successes over the 2012-2013 summer. Two of these masses were small and hatched simultaneously, so they were combined for Experiment 3, cohort OBO (Figure 5.1). All squid were raised from eggs, so they had no post-hatching experience of open water conditions. The squid were housed in two 450 litre vertical cylindrical tanks. The tanks were purpose built from clear acrylic, with a clear acrylic lid. The tanks were 0.8 meter in diameter, and 1.0 meter in height. Both tanks drained into a common 120 litre sump tank which contained the equipment necessary to maintain the water quality, and the water was returned to the tanks in parallel to ensure equal water parameters in the two tanks. A summary of the aquarium set-up and squid husbandry methodology are not directly relevant to this chapter, and can be found in Appendix.
1. One tank was completely covered with a blue filter, the other tank with an orange filter, the details of which are provided in Section 5.4. Three individual experiments were undertaken (Figure 5.1).

![Figure 5.1 - The design of the three experiments conducted. Experiments 1 and 2 used the same squid at different ages; the decrease in n is due to sampling of squid for retinal histology. Experiment 3 used four distinct groups of squid hatchlings, two were combined to create the OBO cohort.](image)

5.2.1. Experiment 1

This first experiment served as a pilot study, and was our first success at hatching and raising squid. Approximately 150 squid hatched on 13/12/2010 from a single large egg mass, and were raised in the tank with the blue filter for 30 days. On day 30, 57 juvenile squid remained. Twenty-eight of the squid were randomly collected and transferred to the orange tank. On day 60, ten squid from each tank were randomly sampled. Measurements taken were dorsal mantle length (Section 5.8) and ocular biometry (Section 5.9). Following biometry measurements, all squid were returned to the tank from which they had been taken.
5.2.2. Experiment 2

The aim of this experiment was to determine the timeliness of change when the optical environments were switched. Due to the difficulty in raising squid to adulthood, the same populations of squid used in Experiment 1 remained in the tanks and were reused for Experiment 2. Experiment 2 also compared photoreceptor histology between the two groups, and also investigated the effect of switching the squid between the two spectral environments (by crossing-over).

On day 84 post-hatching, 10 squid from each tank were randomly sampled and fixed in formalin for histological analysis of their retinas (see Section 5.11). On day 90, the blue tank contained 15 squid, and the orange tank 16 squid. Baseline measurements of dorsal mantle length and ocular biometry were made on all animals, and then all squid were switched to the opposite tank (blue to orange and orange to blue) for the remainder of the experiment. Outcome measures were made on all animals daily for five days following the cross-over.

5.2.3. Experiment 3

The third experiment used three cohorts of squid. The first two cohorts were involved in multiple cross-overs between tanks, while the third remained in the blue tank throughout as a control. This experiment included the measurement of refraction on squid aged 45 and 60 days (see Section 5.10). After day 60, all squid were donated to a local aquarium.

**Cohort OBO (Orange-Blue-Orange sequence)**

Two separate egg masses were combined, and began hatching simultaneously on 8/12/2012, peaking in number at 53 squid (Figure 5.1, Exp 3 OBO). They had been temporarily held in a separate white light tank, but once hatching commenced the hatchlings and egg mass were transferred into the orange tank. At 5 days, the egg mass was removed and squid numbers had stabilised at 5 animals.

Day 30: Measurements of dorsal mantle length and ocular biometry were taken on all 5 squid, which were then transferred from the orange to the blue tank.
Day 45: Measurements of mantle length, ocular biometry and infrared photorefraction were made on the 4 remaining squid. Afterwards, the squid were transferred from the blue tank back into the orange tank.

Day 60: Final outcome measurements were made on 4 squid as for day 45.

**Cohort WOB (White-Orange-Blue sequence)**

Cohort WOB hatched on 19/1/2013, while cohort OBO was still being transferred between the blue and orange tanks. Thirty squid hatched in total, which later stabilised at 4 squid. As we were unsure of the effect of combining squid of different ages in the same tank, they were held in a separate tank with no chromatic filter or temperature regulation (Figure 5.1, Exp 3 WOB) while cohort OBO completed the experiment. As the water was approximately two degrees warmer, this cohort of squid grew appreciably larger than the two other cohorts.

Day 30: Cohort WOB was transferred to the aquarium. Baseline measurements of dorsal mantle length and ocular biometry were made on all 4 squid, which were then placed into the orange tank after allowing acclimatisation to the cooler water temperature.

Day 45: Measurements of dorsal mantle length, ocular biometry and infrared photorefraction were made on all 4 squid. The animals were then transferred from the orange to the blue tank.

Day 60: Final outcome measurements were made on 4 squid as for day 45.

**Cohort BBB (Blue-Blue-Blue sequence)**

A third cohort of eggs began hatching in the blue tank on 4/2/2013, peaking at 12 squid. By day 30, numbers had decreased to 5, then remained at 4 from day 35 (Figure 5.1, Exp 3 BBB). These squid remained in the blue tank as a control for the entire 60 day period with measurements taken every 15 days.

### 5.3. Room lighting

Room lighting was provided by two sets of two ceiling mounted linear fluorescent tubes (Starcoat T5/f28W, General Electric, USA) mounted symmetrically on the ceiling 3 meters above
the tanks. These provided 300 lux at the tank surface, which was one metre above the floor. The bulbs simulated a daylight spectrum, although there were significant peak emissions at 435, 545, and 610nm (data sheet available (GE Lighting 2013)). The lighting cycle in the laboratory was maintained on a 12:12 light:dark cycle for the entire period, with 30 minutes ramp-up and ramp-down (simulating sunrise and sunset).

5.4. Chromatic filter selection

With the exception of *Watasenia scintillans* (Kröger and Gislén 2004), the squid retina contains one visual opsin (Gärtner 2000). The opsin peak sensitivity is towards the blue end of the spectrum, and can be further tuned (by approximately 5nm) to suit the pelagic environment (Messenger 1981; Morris, Bowmaker et al. 1993). *Sepioteuthis sp.* opsin sensitivity has not been reported, and was not measured as part of this thesis. However, opsin sensitivity for another squid that shares a similar surface-to-mesopelagic environment as *Sepioteuthis sp.* has been measured. The *Loligo forbesii* squid retina contains a single opsin, with a peak sensitivity near 494 nanometres (Morris, Bowmaker et al. 1993), and this opsin sensitivity curve was employed for filter selection in this study.

The two study filters (‘blue’ and ‘orange’) used in these experiments were selected to permit light transmittance at opposite ends of the squid opsin sensitivity curve. The spectral sensitivity of squid is extremely poor towards the red wavelengths (Gärtner 2000), so for the longer wavelength filter, good transmittance through the yellow and green was required to ensure adequate total luminance.

The two filters (721 Berry Blue and 179 Chrome Orange, LEE Filters, England) were chosen to exclude each other’s peak wavelengths, while attempting to equalise perceived brightness when matched to the squid opsin sensitivity curve. This was achieved by matching the intercept of the opsin sensitivity and filter transmittance (Figure 5.2). Water acts as a blue filter (see Figure 1B in Warrant and Locket (Warrant and Locket 2004)), however, at a depth of one meter the effect is negligible. The $\lambda_{\text{max}}$ of each filter within the squid opsin sensitivity curve, was 447 and 557nm for the blue and orange filters, respectively.
Calculating the shared area under the curve of the opsin sensitivity and each filter transmittance curve showed the blue tank had 1.16x the luminance of the orange tank. As the radiance in the ocean varies by 100x from the surface to a depth of 50 metres (Warrant and Locket 2004), the cephalopod retina is well adapted to changes in light intensity (Torres, Camacho et al. 1997), and this difference in luminance is likely of little significance in determining the refractive state of the eye.

Figure 5.2 - Comparison of the two experimental filters (721 Berry blue and 179 Chrome orange) transmission curves (provided by the manufacturer, LEE Filters, England) in the lighting used in the lab (Starcoat T5/f28W, General Electric, USA), plotted against a normalised squid spectral sensitivity curve (recreated from Morris et al, Figure 1b (Morris, Bowmaker et al. 1993)).

Once the filters were in place, extreme care was taken to ensure that no white light entered the tanks, until either crossover day or after the conclusion of the experiment. For maintenance work, such as feeding and cleaning, room lights were turned off, and work conducted under either blue or orange torch-light, or under infra-red light (through an infrared sensitive camera). Poor sensitivity to infrared light was verified by a lack of a pupil response, and the absence of a flight reflex from threats illuminated with this light.
5.5. Tank transfers

During transfers between tanks for the cross-over experiments, the unfiltered room lights were dimmed and the squid were collected by submerging a 10 litre bucket in the main tank to catch the squid, and slowly drawing it out. This technique minimally disturbed the squid. When in the new tank, squid were allowed to make their own way out of the bucket. Due to the common water circulating between tanks, no acclimatisation was necessary. Existing food was also transferred to the new tank and replenished to a high density. The new tank was immediately closed to exclude any white light. The squid were monitored until they were either at rest or seen hunting for food.

5.6. Photography of individual squid for measurements

Individual squid were netted and placed into a rectangular glass cell (75l x 25w x 25h mm) assembled from microscope slides, filled with water from the tank at the time of capture (Figure 5.3). The ‘boxed’ squid was transferred into a dark room (<1 lux) and placed under a dissection microscope, on a stand which had been modified to transilluminate the holding cell with infrared LEDs (Kodenshi OPE5794, Tokyo, Japan). In the dark, the squid skin became almost transparent (i.e. their chromatophores were constricted), and the squid simply hovered in the cell, showing no sign of distress. The amount of infrared light passing through the squid could be varied for maximum contrast by adjusting the angle of a reflecting mirror in the light path. The dissection microscope oculars were replaced with a digital high definition video camera with the infrared filter removed (HDR-XR200VE, Sony Electronics, Japan).
Figure 5.3 - The capturing of a single 60 day old squid in the holding cell. Immediately prior to the photo, the only light source was a torch with blue filter, casting only a dim blue light which draws the squid to the surface. A flash has been used in this photo to demonstrate the level of transparency when the squid is in darkness. Visible is the eye cup (<), optic lobe (*), and other internal organs (+).

Six top-down photos of each squid were taken (f/1.8, exposure 1/100s, focal length 20cm) – three at no zoom next to a ruler with millimetre increments for length measurements, and three optically zoomed 4.5x on the eye of the squid. One eye was selected for analysis per squid (stratified so number of left = number of right) determined by the orientation of the squid in the holding cell once captured. The highest quality whole squid and zoomed eye image for each squid (determined by best focus, separation from the edge of the tank, lack of motion blur, and perpendicularity to the camera axis) were selected for analysis.

5.7. Masking of squid origin

Selected images were immediately copied, and the originals put aside. The copied files were stripped of identifying file attributes such as time or tank, and randomly ordered then sequentially renamed. Once analysis was complete, the cleansed images were correlated with their originals based on file size and renamed appropriately.
5.8. Dorsal mantle length measurement

The whole squid image was opened in ImageJ (Version 1.47t, developed by Wayne Rasband, National Institute of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/). The scale was calibrated for each image using the full extent of the visible ruler. One measurement per squid was made from the most posterior point of the mantle, excluding the fin, to the dorsal edge of the mantle hood (Figure 5.4).

![Image of squid showing DML measurement](image)

Figure 5.4 - Measurement of Dorsal Mantle Length (DML). The clearest image from a series of at least 3 images was selected, and a line drawn from posterior pole to the anterior hood of the mantle. The green is false colour applied by the camera during infrared filming. 1mm scale increments can be seen along the bottom of the image.

5.9. Ocular biometry measurement

The optically zoomed image for each squid was cropped to the appropriate eye. Each image was opened in ImageJ, and rotated so that the crystalline lens was orientated down, with the front of the eye cup horizontal (Figure 5.5).
The images were zoomed to 200%, despeckled (a noise-reduction algorithm that replaces each pixel value with the median of the 3x3 surrounding pixels (Health 2013)), and the contrast enhanced to 0.4% black pixel saturation. This heightened the difference between the highly pigmented eye cup and the semi-translucent overlying skin. A minimum of 10 points were marked along the outside edge of the eye cup, concentrating on the posterior pole but including at least 120 degrees. Due to the close proximity of the optic lobe on the lateral side of the eye, the medial side of the eye cup was generally better defined. Using a built-in command in ImageJ, an automated circle of best fit using the Pratt method of least squares was fitted (Vaughan 1987) to the marked points. From this circle, the centre of the retinal circle \((X_R, Y_R)\) and retinal diameter \((D_R)\) was recorded. A similar fitting process was repeated for the visible anterior aspect of the lens, and measurements were collected for the lens (centre of the lens circle \((X_L, Y_L)\), and lens diameter \((D_L)\)). Matthiessen’s Ratio (MR), a method for normalising eye size and removing variation in body size (Kröger and Fernald 1994), was calculated by dividing the retinal diameter by the lens diameter \((D_R / D_L)\). Relative centration of the lens compared to the retinal cup was calculated by determining the displacement of the centre of the lens circle from the centre of the retinal circle \((\langle X_L - X_R \rangle, \langle Y_L - Y_R \rangle)\). To adjust for the x-axis inversion between the left and right eyes, \(X_L\) and \(X_R\) of the left eye was multiplied by -1 to invert the sign, in effect treating all eyes as if they were right eyes. During Experiment One, two independent observers performed analysis separately, and there were only small discrepancies between the mean measurements of squid from each tank between observers. These discrepancies had no impact on the significance, or lack thereof, of each measurement.
5.10. Infrared photorefraction

Infrared eccentric photorefraction was performed in the dark on the boxed squid, in the same eye as used for biometry measurements. The photorefractor design was based on a previous model by Schaeffel (Schaeffel, Farkas et al. 1987). The photorefractor had five rows of infrared LEDs which could be manually operated via a six position switch (Figure 5.6). The first row contained two infrared LEDs, and each subsequent row had an additional LED to compensate for the decrease in brightness due to the eccentricity and the LED half angle of 19°, although this compensation was not formally assessed.

The five row LED array could move vertically along a two-position slider to yield ten eccentricities, ranging from 8.5mm to 55mm. Because of the small eye size, a working distance of 0.4m was used throughout. The LEDs output 20 milliwatts per steradian at 40mA, with a peak

Figure 5.6 - Circuit diagram of the (a) infrared eccentric photorefractor and (b) the camera attachment of the actual product.
wavelength of 940nm and a spectral half-width of 45nm. To increase the light output of the LEDs, the current was increased from 40mA to 80mA (see Figure 5.6) by selecting the nearest (rounded up) 1W resistor necessary to provide each LED with 1.5V at 80mA. Limiting the overdrive to a factor of 2 prevented the shift in peak wavelength that occurs at higher currents (Schaeffel, Farkas et al. 1987), while still increasing light output by an additional 50% (Gardasoft Vision 2008).

The light source was orientated so the LEDs were below the camera aperture, thus giving refractive error along the vertical meridian only. In this orientation, a brighter reflex in the upper part of the pupil indicated relative hyperopia, a brighter lower part of the pupil indicated relative myopia (Roorda, Campbell et al. 1997). An infrared-sensitive high-definition camera (HDR-XR200VE, Sony Electronics, Japan), captured the pupil light reflex profile while all eccentricities were cycled.

Each video was loaded into an open-source video analysis software package (Tracker v4.80, Open Source Physics, USA). The portion of the video which showed the visibly clearest gradient was selected, then the frame number cross-referenced with the original audio recording to obtain the current eccentricity value. A vertical line was down from the top to bottom of the pupil. As the pupil was small (approximately 25 pixels) and the image noisy, the line width was increased to 4 pixels wide and the mean of these four columns was used to create a single pupil light reflex profile for the single frame. To further reduce noise, the pupil light reflex profile was measured and averaged over 5 frames.

A single mean pupil profile, representing a mean of the 4 pixel-wide profile down the pupil over 5 frames of video, was recorded for each squid. A linear regression of the mean pupil profile gave the y-intercept and gradient, which was used to calculate the half-maximum intensity value.
5.7 - Infrared photoretinoscopy of a squid aged 60 days post-hatching. (a) A 4 pixel-wide line (shown in white) is drawn down the centre of the pupil on five frames. (b) The mean of 5 frames were used to create a pupil light reflex profile; along the x-axis ‘0’ is the top of the pupil, and ‘24’ is the bottom. A linear regression of the profile calculated, and the y-intercept and gradient used to calculate the ‘half max’ value. In this example, 11 of the points fall below the calculated half max value (108), which gives a dark fraction value of 45.8%.

The number of pupil profile data points below the half-maximum value divided by the total number of pupil profile data points in the pupil intensity reflex gave the dark fraction (DF) of the pupil. Observation of several squid showed the pupil diameter to be almost as large as the calculated lens diameter, so a value of 98% of the measured lens diameter for each squid was used throughout. Knowing the pupil radius (R), the eccentricity of LED light (E), the testing distance (A), and the dark fraction (DF), the amount of defocus relative to the photorefractor plane (D) could be determined (Bobier and Braddick 1985; Howland 1985):

\[ D = \frac{E}{2 \cdot A \cdot DF \cdot R} \]

The dioptic value was adjusted for the 0.4 metre testing distance by subtracting +2.50D, to give the refraction relative to infinity. The resultant value was divided by 1.34 to adjust for the extra vergence created by the tank : air interface, as is required for underwater refractions (Hueter and Gruber 1980).

5.11. Photoreceptor histology

As part of a joint project with the Department of Anatomy, ten squid from each tank in Experiment 1 were collected at 84 days post-hatching for histological analysis. The animals were
cold anaesthetised, then immediately fixed in formalin. Tissue sectioning and imaging was performed by a technician within the Department of Anatomy. The images and raw data were returned for statistical analysis. A brief summary of the process undertaken by the Department of Anatomy is provided below.

Following fixation, each whole squid was progressively dehydrated in ethanol (70-100% in 10% steps every 24 hours), then rinsed in xylene solution. Each squid was vertically mounted in paraffin wax (sectioning direction arms to mantle) so that a transverse section could obtain both eyes simultaneously. The eye was sectioned down to the visual axis, determined by the maximum diameter of the bisected lens. A 5μm section of retina at the visual axis was warmed in a 49°C water bath, mounted, then baked at 60°C for one hour to remove water residue. The slides were stained with haematoxylin (Gill 2: 4g haematoxylin, 20 mL glacial acetic acid, 70.4g

Figure 5.8 - 5μm thick transverse section of the retina of an 84 day post-hatching squid from the orange tank. The vitreous chamber is on the right of the image. OS indicates the photoreceptor outer-segments; IS the inner segments; and AX the photoreceptor axons which project posteriorly to the eye.
aluminium sulphate, 0.4g sodium iodate, 250mL ethylene glycol, 730mL distilled water) and
eosin (20g EosinY, 2L distilled water, 20mL 1% calcium chloride) to allow easier identification of
individual cells. Photographs of tissue sections were digitally captured at 25-40x magnification
with oil immersion (Leica Type F Immersion liquid oil) using brightfield microscopy (Digital Sight
DS-U1, Nikon, Japan, on DMR upright microscope, Leica Microsystems GmbH, Germany).
Photoreceptor cell density was a count of cell nuclei in three 50µm² blocks: the first directly
behind the lens on the visual axis, and the two neighboring blocks in the same image, one
superior, one inferior. Photoreceptor outer segment length measurements were made from the
margin of the vitreous chamber to the limiting membrane between the outer and inner
segments. Three photoreceptor length measurements were made per eye; one bisecting on axis,
and one 25µm either side of the bisector. A mean of the three measurements was calculated for
each squid. The photoreceptors were prone to histological artifacts which often separated the
inner and outer photoreceptor segments. Only images free from such artifacts were used for
measurements (blue = 5, orange = 8).

5.12. Statistical analysis

Statistical analysis was conducted with SPSS (Version 20, IBM, USA). Normality was tested with
the Shapiro-Wilk test, and equality of variance between measures from the blue and orange
tanks tested with Levene’s Test. Measures with normal distributions and equal variance were
assessed with one-way ANOVA. Adjustments for multiple comparisons were made with Šidák
correction, where necessary. When the assumption of normality was not met, a non-parametric
Mann-Whitney U-test (MWU), or Kruskal-Wallis one-way analysis of variance was conducted.
Linear and orthogonal regressions used a least mean squares method. Comparison of multiple
regressions was conducted with the generalised linear model (GLM). All statistical testing
assumed a significance level of $p = 0.05$. 
Chapter 6. Results: Emmetropisation in *Sepioteuthis australis*

6.1. Experiment 1

6.1.1. Ocular biometry

At 60 days post-hatching, lens diameter ($D_L$) and retinal diameter ($D_R$) were measured on 20 un-anaesthetised squid using infrared transillumination as described in Chapter 5. Ten had been in the blue tank for 60 days from hatching, and 10 had been in the blue tank for 30 days and then in the orange tank for 30 days (see Chapter 5.2.1).

Anatomical measurements from both blue and orange tanks had equal variance for lens diameter (Levene’s, $p = 0.85$) and retinal diameter (Levene’s, $p = 0.85$, Table 6.1). Data from both the blue and orange tanks followed a normal distribution (Shapiro-Wilk, all $p > 0.2$), with the exception of retinal diameter of eyes from the orange tank (Shapiro-Wilk, $p = 0.017$, Figure 6.1a). Manual interpretation of the orange squid retinal diameter histogram and Q-Q plot showed a near normal distribution, which suggests the significant Shapiro-Wilk result is likely due to the small sample size. As a precaution, non-parametric testing was used to compare retinal diameters between the blue and orange tank. A one-way ANOVA was used to compare $D_L$, which showed no significant difference between eyes from the blue and orange tanks (ANOVA, $F_{(1, 18)} = 0.350$, $p = 0.562$). An Independent Samples Mann-Whitney U-test was used to compare retinal diameter due to possible non-normal distribution of data from the orange tank. This test also showed the difference in $D_R$ to be non-significant (MWU, $p = 0.052$).

<table>
<thead>
<tr>
<th></th>
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<th>Mean (mm)</th>
<th>SD</th>
<th>95% CI</th>
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<td>6.11</td>
<td>.363</td>
<td>5.85</td>
<td>6.37</td>
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Table 6.1 - Comparison of biometry measures of: $D_L$ = diameter of the lens, $D_R$ = diameter of the retina.
Figure 6.1 – (a) Distribution of lens diameter ($D_L$), retina diameter ($D_R$), and (b) Matthiessen’s ratio (MR) by tank. Measurements greater than 1.5 times the interquartile range are indicated by circles, greater than three times with *, however all measurements were included in analysis. The difference in MR (#) was significant at the $p = 0.002$ level.

Post-hoc power analysis showed changes in $D_L$ and $D_R$ to have low statistical power in this experiment design, at 11% and 26%, respectively, due to the high variability between individual squid.

6.1.2. Matthiessen’s ratio (MR)

MR in both tanks was normally distributed (Shapiro-Wilk, Blue $p = 0.239$ and Orange $p = 0.419$) and variance between the tanks was equal (Levene’s, $p = 0.922$). Mean MR for the eyes of the squid in the blue tank was significantly smaller than that for eyes from the orange tank ($2.35 \pm 0.07$ vs $2.48 \pm 0.08$, ANOVA, $F_{(1, 18)} = 12.784$, $p = 0.002$, Figure 6.1b, and Table 6.2).

<table>
<thead>
<tr>
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<td>Lower</td>
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<td>Upper</td>
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<tr>
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<td>.221</td>
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<td>.242</td>
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<td>.237</td>
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Table 6.2 - Description of Matthiessen’s ratio (MR) between the blue and orange tanks. Orange had a significantly larger MR than blue ($p = 0.002$).
There was a significant positive correlation between $D_L$ and $D_R$ in both tanks (Pearson’s $r$, 2-tailed, Blue $r = 0.837$, $n = 10$, $p = 0.003$ and Orange $r = 0.846$, $n = 10$, $p = 0.002$). There was no significant correlation of MR with $D_L$ or $D_R$ in either tank (Pearson’s $r$, 2-tailed, $p > 0.05$, Table 6.3).

<table>
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<tr>
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<th>$D_L$</th>
<th>$D_R$</th>
<th>MR</th>
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<tr>
<td>Blue</td>
<td>Pearson r</td>
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<td>$p$ (2-tailed)</td>
<td><strong>0.003</strong></td>
<td>0.529</td>
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<td>Pearson r</td>
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<td></td>
<td>$p$ (2-tailed)</td>
<td><strong>0.002</strong></td>
<td>0.915</td>
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Table 6.3 - Pearson correlations between lens diameter ($DL$), retinal diameter ($DR$), and Matthiessen’s ratio (MR). Significant correlations are indicated in bold.

Post-hoc power analysis showed high statistical power of MR at 94%, as the normalisation of the retina to the lens removed the high variability in absolute size between squid.

6.1.3. Lens position

It was possible that the squid eye might have compensated for the different focal lengths created by the different spectral environments by displacing the centre of the lens relative to the centre of the retina. If this were the case, we would expect lenses of squid from the blue tank to be posteriorly (greater $Y_L$, along the optical axis) or laterally (greater absolute $X_L$, normal to the optical axis) displaced relative the retinal centres, than the lenses of squid from the orange tank.

All measures of lens position (lens centre relative to retinal centre) were normally distributed (Shapiro-Wilk, $p > 0.5$) with the exception of $X_L$ in the orange tank ($p = 0.18$). Examination of the Q-Q plot revealed the deviation from normal in the orange tank was due to a single point measuring higher than expected. Testing confirmed this point as an outlier (Grubb’s, $\alpha = 0.05$, $Z = 2.36$). However, as removing this point did not affect the resulting $p$-value, it was not excluded. The distribution of lens position (lens centre relative to the retinal circle centre) was equal between tanks for both medial-lateral ($X_L$) and anterior-posterior ($Y_L$) displacement (Levenes, $X_L$, $p = 0.520$, $Y_L$, $p = 0.77$). Relative to the retinal centre, the position of the lens centre
in squid eyes from both the blue and orange tanks was laterally displaced (0.05mm vs 0.06mm, respectively), and there was no significant difference in lateral displacement between tanks (MWU, \( p = 0.853 \), Table 6.4 and Figure 6.2).

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Mean (mm)</th>
<th>SD</th>
<th>95% CI</th>
<th>Minimum</th>
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<td></td>
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<td>Upper</td>
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<td>.0801</td>
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<td>.0697</td>
<td>.009</td>
<td>.108</td>
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<td>( Y_L )</td>
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<td>.1185</td>
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<td>.195</td>
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<td>-.059</td>
<td>.1909</td>
<td>-.195</td>
<td>.078</td>
</tr>
</tbody>
</table>

Table 6.4 - Displacement of the lens centre relative to the retinal centre along the medio-lateral (\( X_L \)) and antero-posterior axis (\( Y_L \)). A negative number indicates the lens centre is medial or anterior.

Analysis of the anterior-posterior displacement along the optical axis (\( Y_L \)) revealed the lens centre for squid eyes from the blue tank to be more posterior (0.11 ± 0.19mm) compared with squid eyes from the orange tank (-0.06 ± 0.19mm), whose lens centre was slightly anterior to the centre of the retina, increasing the distance from the retina. Parametric means testing showed the difference in \( Y_L \) between tanks to be significant (ANOVA, \( F_{(1,18)} = 3.521, p = 0.029 \)).

Testing for deviation of the lens centre relative to the retinal centre showed no significant lens displacement of \( X_L \) in either tank, however mean \( Y_L \) for eyes from the blue tank was significantly posteriorly displaced relative to the retinal centre (Sidak adjusted \( p = 0.025 \) 1-sample T-test, \( t = 2.951, p = 0.016 \)).
6.1.4. Dorsal mantle length

The dorsal mantle length (DML) of squid from the blue and orange tanks were normally distributed (Shapiro-Wilk, $p > 0.5$) and had equal variance (Levene's, $p = 0.922$). Only nine images (out of ten) from the orange tank were suitable for analysis. All ten from the blue tank were suitable. DML of squid from the orange tank was slightly, but significantly, longer than DML of squid kept in the blue tank ($22.77 \pm 1.18 \text{mm vs } 21.41 \pm 1.11 \text{mm}$, ANOVA, $F_{(1,17)} = 6.734$, $p = 0.019$, Figure 6.3a).

While squid in the orange tank were both longer overall and had larger MR, there was no significant correlation between DML and MR in squid in either the blue or orange tank (Pearson 2-tailed, Blue $r = -0.269$, $n = 10$, $p = 0.451$ and Orange $r = -0.105$, $n = 9$, $p = 0.788$, Figure 6.3b).
6.2. Experiment 2

Thirty-one 90 day old squid were used for the crossover experiment. Baseline measures were made on 16 squid from the blue tank (having been in the blue tank for 90 days from hatching), and 15 squid from the orange tank (having been in the blue tank for 30 days, then in the orange tank for 60 days). The squid were switched between tanks immediately after baseline measurements, and the numbers of squid remained constant throughout the five day follow up (15 in blue, 16 in orange). Outcome measures were taken daily for five days following crossover (until more food had to be procured), with the first tank on which outcome measures were made alternated each day to reduce measurement bias.

6.2.1. Ocular biometry

Mean lens diameter ($D_L$) was not different between squid from the blue and orange tanks at baseline (Table 6.5). Lenses from both tanks increased in size over the five days for which they were followed. However, lens size was not significantly different between tanks at any stage. Lens growth appeared linear over the five day period (blue 0.031mm/day, $R^2 = 0.965$; orange 0.029mm/day, $R^2 = 0.837$), and the growth rate was not significantly different between tanks (Repeated Measures GLM, $F_{(1)} = 0.268, p = 0.608$, Figure 6.4).
Retinal diameter ($D_R$) was not significantly different between squid from the blue and orange tank at baseline (Table 6.5). The increase in $D_R$ over the five day period was linear in both tanks (blue $0.086 \text{mm/day, } R^2 = 0.771$; orange $0.089 \text{mm/day, } R^2 = 0.789$). While the daily increase in $D_R$ in each tank was not equal during the five day follow up (Figure 6.4), the difference in $D_R$ between the blue and orange tank was not significant at any stage (Repeated Measures GLM, $F_{(1)} = 0.131, p = 0.720$).

![Figure 6.4](image)

**Figure 6.4 -** Growth of lens diameter ($D_L$) and retinal diameter ($D_R$) during the crossover period. $D_L$ was initially equal at baseline, all subsequent measurements of $D_L$ were less in the orange than blue tank, indicating lower growth, although no significant difference in $D_L$ was noted between tanks at any time. $D_R$ was more variable and both tanks increased by approximately 0.1mm per day. Error bars are SEM.

<table>
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<th>48</th>
<th>72</th>
<th>96</th>
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<tr>
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<tr>
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<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Orange</td>
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<td><strong>Lens diameter (mm)</strong></td>
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<tr>
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Table 6.5 - Comparison of the mean (Mn) $D_L$ and $D_R$ at baseline (BL) and over the five daily measurements after filter crossover. No significant differences were noted between the tanks. $D_L = \text{diameter of the lens, } D_R = \text{diameter of the retina.}$
6.2.2. Matthiessen’s ratio

Unlike Experiment 1, the MR of squid from the blue and orange tank were not significantly different in these 90 day old squid at baseline (ANOVA, $F_{(1,29)} = 3.445, p = 0.074$, Table 6.6). Compared to the 60 day old squid measurements in Experiment 1, MR had decreased at the 90 day baseline measurement in both the blue ($2.35 \pm 0.07$ to $2.28 \pm 0.19$) and the orange tank ($2.48 \pm 0.08$ to $2.38 \pm 0.11$). However, over the five day period following crossover, MR in the squid transferred into the blue tank decreased from $2.38 \pm 0.11$ to $2.26 \pm 0.10$, whereas the MR of squid transferred into the orange tank increased from $2.28 \pm 0.19$ to $2.33 \pm 0.12$.

Following crossover, apparent equality in mean MR for eyes from the blue and orange tanks had occurred by 48 hours (Figure 6.5). However, MR for eyes from the blue and orange tanks was not significantly different at any single outcome measurement point following the crossover (ANOVA, all $p > 0.05$, Table 6.6). The decrease in MR for squid eyes transferred into the blue tank was significantly different between baseline and 120 hours post-crossover (Paired t-test, $t_{(14)} = 2.442, p = 0.028$, 2-tailed). However the increase in MR for squid eyes transferred into the orange tank failed to reach significance (Paired t-test, $t_{(15)} = -1.093, p = 0.292$, 2-tailed). A general linear model of the effect of each spectral environment on the change in MR over the five day period showed a significantly different effect between cohorts (Repeated measures GLM, $F_{(1)} = 6.820, p = 0.014$).

<table>
<thead>
<tr>
<th></th>
<th>BL</th>
<th>24hr</th>
<th>48hr</th>
<th>72hr</th>
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<tr>
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Table 6.6 – Mean (Mn) Matthiessen’s ratio (MR) in the two cohorts of squid at baseline (BL) and following crossover. The colour indicates the tank the squid had been in prior to the measurement; the crossover occurred immediately after the baseline measurement. The only significant difference between measurements is indicated in bold.
6.2.3. Lens position

Linear regression showed no significant trend in either $X_L$ or $Y_L$ over time in either tank ($X_L$: blue $R^2 = 0.345$, $p = 0.146$; orange $R^2 = 0.179$, $p = 0.268$. $Y_L$: blue $R^2 = 0.182$, $p = 0.285$; orange $R^2 = -0.453$, $p = 0.960$). The change in $X_L$ and $Y_L$ from baseline was not significantly different in squid from either the blue (ANOVA, $F_{(6,8)} = 2.229$, $p = 0.146$) or orange tank (ANOVA, $F_{(6,9)} = 1.544$, $p = 0.268$) at any time.

6.2.4. Dorsal mantle length

Squid transferred from the orange into the blue tank were initially larger than those transferred from the blue into the orange tank, and they remained so for the entire period of five days. Growth of squid in both tanks was approximately linear (blue 0.423mm/day, $R^2 = 0.797$; orange 0.502mm/day, $R^2 = 0.917$, Figure 6.6), and the difference in growth rate of squid between the
blue and orange tank was not significantly different (Repeated measures GLM, $F_{(1)} = 0.055, p = 0.816$).

![Figure 6.6 - Dorsal mantle length (DML) of squid in the orange and blue tanks. There was no significant difference of DML between squid from the blue and orange tanks at any point. Both tanks appeared to show an initial plateau of growth immediately after the crossover. Error bars SEM.](image)

### 6.2.5. Retinal histology

Twenty squid, ten from the blue and ten from the orange tank, were sampled and processed for histology prior to the crossover experiment. Photoreceptor cell density in retinas of squid from the blue tank (Table 6.7) was not significantly different from that of squid from the orange tank (ANOVA, $F_{(1,18)} = 0.407, p = 0.531$). Nor was total photoreceptor length significantly different in the retinas from the blue and orange tanks (ANOVA, $F_{(1,18)} = 0.351, p = 0.561$). There was also no significant difference in the photoreceptor outer segment lengths between eyes from the two tanks (ANOVA, $F_{(1,18)} = 0.109, p = 0.745$).
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Table 6.7 - Cell density, photoreceptor outer segment (OS) length, and total photoreceptor (PR) length of squid from the blue and orange tank. No significant differences between the tanks were noted.

6.3. Experiment 3

In total, 14 squid were divided into three cohorts and exposed to a series of spectral environments; white light (W), blue light (B), or orange light (O). Three different lighting paradigms were investigated: WOB, OBO, and BBB as a control cohort (see Chapter 5.2.3). Outcome measures were made on day 30, day 45 and day 60.

6.3.1. Ocular biometry

Lens diameter (Dₐ) increased in size from day 30 to day 60 in all cohorts (Table 6.8). There was a significant difference in Dₐ between cohorts at day 30 (ANOVA, F(2,11) = 54.368, p < 0.0001, Table 6.9); post hoc testing with the Tukey HSD test showed eyes from the white tank (WOB cohort) to have significantly larger Dₐ (1.42 ± 0.05mm) than those from both the blue tank (BBB cohort) (1.03 ± 0.09mm, p < 0.0001) and orange tank (OBO cohort) (0.98 ± 0.06mm, p < 0.0001). At day 45, there was no significant difference in Dₐ from any cohort (ANOVA, F(2,10) = 0.443, p = 0.654). However, on day 60 there was once again a difference in Dₐ (ANOVA, F(2,9) = 20.607, p = 0.0004). Tukey HSD showed the WOB cohort to have significantly larger Dₐ (2.63 ± 0.11) than both the OBO (1.82 ± 0.17, p < 0.0001) and BBB cohorts (2.14 ± 0.20, p = 0.0003).
Day 30 & Day 45 & Day 60
\hline
\textbf{D_L} (mm) & \textbf{BBB} & 5 & 1.03 & 0.09 & 5 & 1.58 & 0.10 & 4 & 2.14 & 0.20 \\
& \textbf{OBO} & 5 & 0.98 & 0.06 & 4 & 1.60 & 0.18 & 4 & 1.82 & 0.17 \\
& \textbf{WOB} & 4 & 1.42 & 0.05 & 4 & 1.66 & 0.11 & 4 & 2.63 & 0.11 \\
\textbf{D_R} (mm) & \textbf{BBB} & 5 & 2.44 & 0.11 & 5 & 3.67 & 0.34 & 4 & 4.93 & 0.14 \\
& \textbf{OBO} & 5 & 2.71 & 0.14 & 4 & 3.67 & 0.07 & 4 & 4.52 & 0.04 \\
& \textbf{WOB} & 4 & 3.44 & 0.17 & 4 & 4.54 & 0.17 & 4 & 5.74 & 0.28 \\
\hline

Table 6.8 - Mean and standard deviation of ocular biometry measurements by squid cohort at day 30, 45, and 60. Filter colour at the time of measurement is indicated with the cell colour; no filter, orange, or blue. \(D_L\) = lens diameter, \(D_R\) = retina diameter.

At day 30, retinal diameter (\(D_R\)) was significantly different between cohorts (ANOVA, \(F_{(2,11)} = 62.016, p < 0.0001\), Table 6.8 and Table 6.9). \(D_R\) of squid from the white tank was larger (WOB, 3.44 ± 0.17mm) than \(D_R\) of squid from the blue (BBB, 2.44 ± 0.11mm, \(p < 0.0001\)) and orange (OBO, 2.71 ± 0.14mm, \(p = 0.025\)) tanks. This remained true at day 45, after squid which had previously been in the white tank (WOB) had been moved to the orange tank (ANOVA, \(F_{(2,10)} = 13.324, p = 0.002\)). \(D_R\) in the WOB cohort remained larger than in the OBO and BBB cohorts at day 60 (ANOVA, \(F_{(2,9)} = 35.955, p < 0.0001\)). There was no significant difference in \(D_R\) between OBO and BBB cohorts at day 30 or 45. However, at day 60 the BBB cohort had a significantly larger \(D_R\) than the OBO cohort (4.93 ± 0.14mm vs 4.52 ± 0.04mm, \(p = 0.046\)).

\begin{tabular}{|c|c|c|c|c|c|}
\hline
\textbf{df} & \textbf{F} & \textbf{Sig.} \\
\hline
\textbf{D_L} & 2 & 54.914 & <0.0001 \\
\textbf{D_R} & 2 & 61.224 & <0.0001 \\
\hline
\textbf{df} & \textbf{F} & \textbf{Sig.} \\
\hline
\textbf{D_L} & 2 & 0.458 & 0.645 \\
\textbf{D_R} & 2 & 13.084 & 0.002 \\
\hline
\textbf{df} & \textbf{F} & \textbf{Sig.} \\
\hline
\textbf{D_L} & 2 & 20.498 & 0.0004 \\
\textbf{D_R} & 2 & 36.264 & <0.0001 \\
\hline
\end{tabular}

Table 6.9 - ANOVA of \(D_L\) and \(D_R\) across the three groups of squid over day 30, 45, and 60. Significant differences are indicated in bold. \(D_L\) = lens diameter, \(D_R\) = retinal diameter. Post-hoc testing with the Tukey-HSD is described in the main text.
6.3.2. Matthiessen’s ratio

For all outcome measures, MR generally changed in the direction predicted by the different focal lengths created by LCA (Figure 6.7); MR being higher in squid from the orange tank, and lower in squid from the blue tank.

![Graph showing change of MR over time in the three groups.](image)

Figure 6.7 - Change of MR over time in the three groups. Each cohort was in either white (W), blue (B), or orange (O) spectral environments for 15 days preceding each measurement, and then the environment was changed. OBO, WOB, and BBB, indicate the order of lighting environments for each cohort of squid for the three measurement periods. See text below for significant differences. Error bars SEM.

6.3.2.1. Within cohort analysis

Within the OBO cohort, MR changed significantly over the three measurements (ANOVA, $F_{(2, 10)} = 6.230, p = 0.017$). Post hoc comparison with the Tukey HSD test showed MR at day 30 ($2.78 \pm 0.18$) was significantly different from the mean at day 45 ($2.32 \pm 0.17, p = 0.015$). However, MR at day 60 ($2.54 \pm .27$) was not significantly different from either day 30 ($p = 0.144$) or day 45 ($p = 0.412$).

The WOB cohort also showed a significant difference in MR over the three measurements (ANOVA, $F_{(2, 9)} = 26.831, p = 0.0002$). Post hoc comparison showed a significant difference
between all measurements, including from day 30 (2.42 ± 0.65) to day 45 (2.75 ± 0.17, \( p = 0.005 \)), and day 60 (2.18 ± 0.67, \( p = 0.0001 \)), and between day 45 and day 60 (\( p = 0.036 \)).

The BBB control cohort appeared to show a decreasing MR over time (2.37 ± 0.12, 2.34 ± 0.16, 2.32 ± 0.19 at day 30, 45, and 60, respectively). However the means on each measurement day were not significantly different (ANOVA, \( F_{(2,11)} = 0.178, p = 0.840 \)).

### 6.3.2.2. Between cohort analysis

At day 30, MR between cohorts was significantly different (ANOVA, \( F_{(2,11)} = 12.355, p = 0.002 \)). Tukey HSD showed that MR for the squid in the orange tank (OBO, 2.78 ± 0.18) was significantly larger than MR for both the white light (WOB, 2.42 ± 0.65, \( p = 0.006 \)) and blue control cohorts (BBB, 2.37 ± 0.12, \( p = 0.002 \)). However, MR for WOB and BBB cohorts were not significantly different (\( p = 0.927 \)).

At day 45, MR was again significantly different between cohorts (ANOVA, \( F_{(2,10)} = 8.732, p = 0.006 \)). The squid that were moved from white light into the orange tank now had a higher MR (WOB, 2.75 ± 0.17) than squid moved from orange into the blue tank and also the blue control cohort (OBO, MR = 2.32 ± 0.20, \( p = 0.010 \) and BBB, 2.35 ± 0.14, \( p = 0.013 \), respectively). MR of squid from the OBO and BBB cohorts, which at day 45 had both been in the blue tank for 15 days, were not significantly different from each other (\( p = 0.931 \)).

At day 60, the squid which had been moved back into the orange tank (OBO) for 15 days had an increased MR, and those moved into the blue tank (WOB) had a decreased MR. The difference in MR at day 60 approached, but failed to reach a significance difference between any group (ANOVA, \( F_{(2,9)} = 3.169, p = 0.091 \)) at this time period (Figure 6.7).

### 6.3.3. Lens position

There was little variation in \( X_L \) and \( Y_L \) between cohorts, or over time within each cohort. The position of the lens centre relative to the retinal centre for each cohort at each measurement period is presented in Table 6.10.
<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
<th>95% CI</th>
<th>Minimum</th>
<th>Maximum</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Lower</td>
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<td></td>
<td></td>
<td></td>
<td>Upper</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>OBO</strong></td>
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<tr>
<td>$X_L$</td>
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<td>0.094</td>
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<td>45</td>
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<td>0.0832</td>
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<td></td>
<td>60</td>
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<td>0.130</td>
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<td>0.209</td>
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<tr>
<td>$Y_L$</td>
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<td><strong>WOB</strong></td>
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<td></td>
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</tr>
<tr>
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<td></td>
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<td>0.063</td>
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Table 6.10 - Position of the lens centre ($X_L$, $Y_L$) relative to the centre of the retina of squid at day 30, 45, and 60. Current tank is indicated by colour. Negative values indicate a medially ($X_L$) or anteriorly ($Y_L$) displaced lens.

### 6.3.3.1. Within cohort analysis

$X_L$ and $Y_L$ were not significantly different over the three measurement periods (30, 45, and 60 days post-hatching) in any of the three cohorts (OBO, WOB, BBB, Table 6.11), indicating the mean lens centre was not significantly altered after a cohort of squid was moved into a new spectral environment.
### Table 6.11 - One-way ANOVA of $X_L$ (medio-lateral lens displacement) and $Y_L$ (anterio-posterior lens displacement) over the three measurement periods (Day 30, 45, and 60) by group (OBO, WOB, and BBB, indicating their tanks for each measurement period: W = white, B = blue, O = orange).

<table>
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<tr>
<th></th>
<th>$X_L$</th>
<th>$Y_L$</th>
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<tbody>
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<td>OBO</td>
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<tr>
<td>Between Groups</td>
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<tr>
<td>Within Groups</td>
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<tr>
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<tr>
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</tr>
<tr>
<td>BBB</td>
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</tr>
<tr>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Sum of Squares</th>
<th>df</th>
<th>$F$</th>
<th>$p$</th>
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<td>.856</td>
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<tr>
<td>Within Groups</td>
<td>.081</td>
<td>.161</td>
<td>.854</td>
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<tr>
<td></td>
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</tr>
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<td>.166</td>
</tr>
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<td>Within Groups</td>
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</tr>
<tr>
<td></td>
<td>.166</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### 6.3.3.2. Between cohort analysis

On day 30 there was no significant difference in $X_L$ between cohorts (ANOVA, $F_{(2,11)} = 0.247, p = 0.785$). However, there was a significant difference in $Y_L$ (ANOVA, $F_{(2,11)} = 5.452, p = 0.023$). Post hoc testing with Tukey HSD showed squid from the white tank (WOB, $-0.166 \pm 0.131$mm) to have anteriorly displaced lens centres compared with both squid from the orange (OBO, $0.004 \pm 0.042$mm, $p = 0.048$) and blue (BBB, $0.025 \pm 0.096$mm, $p = 0.027$) tanks.

At day 45, there was no significant difference in $X_L$ (ANOVA, $F_{(2,10)} = 0.813, p = 0.471$) or $Y_L$ (ANOVA, $F_{(2,10)} = 0.286, p = 0.757$). On day 60, again there was no significant difference in $X_L$ (ANOVA, $F_{(2,9)} = 0.261, p = 0.776$) or $Y_L$ (ANOVA, $F_{(2,9)} = 0.815, p = 0.473$).

#### 6.3.4. Photorefraction

Photorefraction measures were made on all three cohorts of squid (WOB, OBO, and BBB), but due to eye size limitations, only measurements at 45 and 60 days post-hatching could be...
analysed. Refractions presented were measured using infrared light with no correction made for wavelength. However, refractions have been corrected for the distance of the photorefractor from the eye, and the effect of measuring refraction underwater through the tank: air interface (Hueter and Gruber 1980).

6.3.4.1. Within cohort analysis

Squid from both OBO and BBB cohorts showed a significant change in refraction between 45 and 60 days post-hatching (Table 6.12). However, there was no significant change in refraction in squid from the WOB cohort after moving from the orange to blue tank.

<table>
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<th>Day 60</th>
<th></th>
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</thead>
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<td>Mean</td>
<td>SD</td>
<td>n</td>
</tr>
<tr>
<td>BBB</td>
<td>5</td>
<td>19.40</td>
<td>1.750</td>
<td>4</td>
</tr>
<tr>
<td>OBO</td>
<td>4</td>
<td>17.16</td>
<td>2.360</td>
<td>4</td>
</tr>
<tr>
<td>WOB</td>
<td>4</td>
<td>13.25</td>
<td>1.963</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 6.12 – The change in mean refraction of each cohort of squid between 45 and 60 days post-hatching. The filter colour of the tank at the time of measurement is indicated by colour. Refractions presented have been corrected for the tank: air interface, but have not been corrected for measurement with infrared wavelength. Refraction could not be measured at 30 days post-hatching due to the small eye size of the squid. Significant differences are indicated in bold.

6.3.4.2. Between cohort analysis

In general, squid from the blue tank had more hyperopic refractions than those from the orange tank (Figure 6.8).

At day 45, there was a significant difference in refraction between cohorts (ANOVA, $F_{(2,10)} = 10.480, p = 0.004$). Tukey HSD testing showed the squid from the orange tank (WOB, $13.25 \pm 1.96D$) to have significantly less hyperopic refractions than the squid from the blue tank (OBO, $17.16 \pm 2.36D, p = 0.049$) and the control blue group (BBB, $19.40 \pm 1.75D, p = 0.003$). The refractions of the two cohorts of squid in the blue tank at 45 days post-hatching (OBO and BBB) were not significantly different from each other ($p = 0.268$).
At day 60, again there was a significant difference in refraction between cohorts (ANOVA, \(F_{(2,9)} = 5.309, p = 0.03\)). Post hoc testing showed the WOB cohort refractions (16.04 ± 2.76D) to be significantly more hyperopic than the OBO cohort (11.38 ± 1.66D, \(p = 0.028\)), but not significantly different from the control BBB cohort (14.74 ± 1.66D, \(p = 0.674\)). Refractions of squid from BBB and OBO were not significantly different at 60 days post-hatching (\(p = 0.111\)).

6.3.4.3. Matthiessen’s ratio and photorefraction correlation

Squid eyes from the blue tank tended to have lower MR and higher refractions than squid from the orange tank (Figure 6.9). However, there was no significant correlation between MR and refraction (Pearson 2-tailed, \(R = -0.321, n = 25, p = 0.117\))
Figure 6.9 - Distribution of Matthiessen's ratio (MR) and photorefraction (D), labelled by tank. The orthogonal regression y-intercept is 2.75, and the gradient -0.023D/MR. Pearson 2-tailed testing showed no significant correlation between MR and photorefraction (R = -0.321, p = 0.117).

6.3.5. Dorsal mantle length

There was a significant difference in DML on day 30 (ANOVA, $F_{(2,11)} = 10.506, p = 0.003$), and post hoc testing showed the squid in the white tank (WOB, 14.08 ± 1.57mm) to be significantly larger than those in blue control tank (BBB, 10.90 ± 1.12mm, $p = 0.004$) and those in the orange tank (OBO, 11.02 ± 0.74mm, $p = 0.006$). On day 45, a difference remained (ANOVA, $F_{(2,10)} = 8.178, p = 0.008$) but now only squid from WOB (20.42 ± 1.56mm) and OBO (15.46 ± 0.68mm, $p = 0.08$) were significantly different. On day 60, DML was significantly different (ANOVA, $F_{(2,9)} = 25.883, p = 0.0002$), with the squid from the WOB cohort (27.15 ± 0.93mm) being significantly larger than squid from OBO (20.83 ± 2.02mm, $p = 0.0002$) and BBB cohorts (22.00 ± 0.90mm, $p = 0.001$). The OBO and BBB cohorts did not have significantly different DML at any of the outcome measures.
Chapter 7. Discussion: Emmetropisation in *Sepioteuthis australis*

The results presented in Chapter 6 suggest that the squid eye emmetropises, and is able to regulate its growth in either direction in response to a manipulated focal plane. As the last common ancestor with vertebrates lacked a camera-type eye, this suggests that emmetropisation has evolved at least twice. Experiment 1 shows that after squid are transferred into an orange spectral environment, which due to intrinsic longitudinal chromatic aberration (LCA) decreases the effective power of the squid crystalline lens, the normalised eye size tends to increase relative to squid kept in a blue spectral environment. Experiment 2 shows that this response occurs rapidly, within 48 hours. Experiment 3 shows the emmetropisation mechanism to be flexible, and can rapidly reverse after multiple adjustments of the focal plane.

### 7.1. Matthiessen’s ratio

In Experiment 1, squid were raised to 30 days post-hatching in the blue tank, and then half of them were transferred into the orange tank. At 60 days post-hatching, ten squid from each tank were sampled. While there was no significant difference between squid from the blue or orange tank in either the diameter of the lens or the retina, when the retinal diameter was compared as a ratio of the lens diameter (Matthiessen’s ratio, MR), the squid from the orange tank had significantly larger MR. Squid moved from the blue to the orange tank, showed an increase in MR in response to the increased focal length compared to those remaining in the blue (2.48 ± 0.078 versus 2.35 ± 0.073, \( p = 0.002 \)). Experiment 2 demonstrated that regulation of eye growth via changes in MR can occur in response to both imposed myopic and hyperopic defocus. Prior to this experiment, squid aged 90 days post-hatching had been in either the blue or orange tank for at least 30 consecutive days. The spectral environments were switched so the squid that were previously in the blue tank were now in the orange tank (simulating hyperopic defocus), and those squid previously in the orange tank were now in the blue tank (simulating myopic defocus). After 120 hours, the squid in the blue tank had a significantly smaller MR compared to baseline (2.38 ± 0.11 to 2.26 ± 0.10, \( p = 0.028 \)), however the squid placed into the orange tank failed to show a significant increase in MR from baseline (2.28 ± 0.19 to 2.33 ± 0.12, \( p = 0.292 \)).
This could indicate a difference in the strength of response between myopic and hyperopic defocus, with the ability of the eye to ‘halt’ growth in response to a myopic stimulus being greater than the stimulus needed to increase relative retinal growth in the presence of hyperopic defocus, at least over a period of 120 hours. In Experiment 3, where the spectral environment crossover occurred in younger squid (between 30 and 60 days post-hatching), the change in MR in both directions was more pronounced, and significant changes in MR were seen in both directions (blue to orange, and orange to blue).

Therefore, as has been demonstrated in many vertebrates (guinea pig (Howlett and McFadden 2006), marmoset (Troilo and Judge 1993), rhesus macaques (Smith III and Hung 1999), tree shrew (Norton and McBrien 1992)), avians (Wallman, Adams et al. 1981), and fish (Kröger and Wagner 1996), the squid eye appears to possess an emmetropisation mechanism, and is capable of regulating its eye growth in either direction.

MR (sometimes referred to as the ‘focal ratio’) is commonly used to discuss the light gathering ability of the eye, or as a measure to compare eye size between marine species (Femald 1990). A similar measure (Nasal-temporal diameter / lens radius, which assuming a hemispherical retina, would give double the MR value) has previously been used to compare the relative retinal size of Aequidens pulcher (Blue Acara) raised in different spectral environments (Kröger and Wagner 1996). In a related fish, Haplochromis burtoni (African cichlid), the correlation of the diameter of the retina to the diameter of the lens produces a stronger correlation than when the retina is normalised to body size, due to variations in allometric growth (Kröger and Fernald 1994). MR of laboratory raised S. lessoniana and S. officinalis (cuttlefish) aged between 4 weeks to 10 months ranged from 2.33 to 2.80, although a high number of lens opacities were noted, which may affect normal refractive development. Another measure of MR in large (20cm+) S. officinalis showed values of 2.11 and 2.12 in two animals. Therefore, the range of MR seen in Experiments 1, 2, and 3 seems to agree with the previously reported literature, and suggest that the effect of the two different spectral environments did not make the eyes abnormally proportioned.

7.1.1. Matthiessen’s ratio over time

Interestingly, despite Experiment 2 using the same population of squid as Experiment 1, no significant difference in MR was seen between squid from the blue and orange tank at Experiment 2 baseline. At Experiment 2 baseline, mean MR in squid from both the blue and
orange tanks had decreased between day 60 to day 90 (blue: 2.35 ± 0.073 to 2.28 ± 0.19, orange: 2.48 ± 0.078 to 2.38 ± 0.11). The control cohort in Experiment 3 (BBB) showed a trend of decreasing MR over time between 30 and 60 days, despite no alterations to the focal plane (see figure zz (add once combined)). This trend can also be seen when comparing MR from Experiment 1 and 2, and also when comparing MR in Experiment 3 between 30, 45, and 60 days post-hatching.

Such decreases in MR with growth have been reported in other species (sparidae fish (Shand, Døving et al. 1999), cichlid fish (Kröger and Fernald 1994), lungfish (Bailes, Trezise et al. 2007), skate (Sivak and Luer 1991), and sharks (Litherland, Collin et al. 2009)). One advantage of a high MR at birth is increased depth of focus; this may provide adequate vision until emmetropisation has reduced refractive error (Shand, Døving et al. 1999). As MR decreases, the f-stop (lens focal length / diameter of the entrance pupil) of the eye increases, which reduces the depth of focus, and increases the light sensitivity of the eye (Jagger and Sands 1999; Sale 2002). In combination with a larger overall eye size, which also increases its light capture abilities (Warrant and Locket 2004; Nilsson, Warrant et al. 2012), the decrease in MR seems well suited to the *S australis* life cycle, in which it is born coastally, but ventures into greater, darker, depths in adulthood (Triantafillos 2001).

### 7.2. Lens or retina?

Matthiessen’s ratio is composed of just two measures: the lens diameter and the retinal diameter. While MR was significantly different, neither the lens nor retinal diameter was significantly different between squid from the two spectral environments in Experiment 1. Additionally, during Experiment 2, there was no significant difference in either the lens or retinal diameter growth rates between the two spectral environments over the five days of follow-up post crossover. As there are only two optical components comprising the squid eye; namely the power of the crystalline lens and the focal length provided by the size of the retina, it is curious why neither was significantly different between the cohorts of squid in different spectral environments.
Squid eye growth post-hatching has not been previously detailed, but as *S. australis* mantle length at hatching can vary by almost a factor of two (Steer, Pecl et al. 2003), and growth rates after hatching are highly variable within a population (Pecl 2004), presumably variation in absolute eye size also occurs. Therefore, it is probable the true change in size of the lens and retina was lost in the noise created by this natural size variability. To increase the power of the experiment, a larger sample size, or a method of identifying individual squid, would be required. Support for the loss of signal arises from the significant difference in MR between squid from the blue and orange spectral environments. If the assumption that larger eyes would have larger lenses is true, by creating a ratio of the lens and retinal diameters (MR), the variability in absolute squid eye size is reduced.

In fish, which have the same eye optics as squid (neutralised cornea, single refractive lens), changes in the refractive index of the crystalline lens can occur in response to visual input, such as a change in the luminance level (Gagnon, Shashar et al. 2011; Kröger 2013). The lens can change its optical properties rapidly, even in the lens nucleus where cells no longer contain functional organelles (Schartau, Kröger et al. 2010). It is possible that changes to the properties of the squid lens (e.g. refractive index) occurred when changing between spectral environments, other than just a change in diameter. The change in the properties of the fish lens may be regulated by dopamine, as when dopamine is deprived or blocked with an antagonist, the lenticular changes are similar to a dark adapted state (Schartau, Sjögreen et al. 2009; Schartau, Kröger et al. 2010). Dopamine is also present in cephalopods (Messenger 1996), and it has an inhibitory effect in the optic lobes (Chrachri and Williamson 2005), however the effect of dopamine on the cephalopod lens has not been investigated.

Fish also provide evidence that the retina can adjust its growth in response to visual input. Significant changes in retinal diameter have also been observed in fish. When one eye of *Oreochromis niloticus* (Tilapia) is deprived of form vision for 28 days, the axial length becomes significantly longer than the control eye, and the eye is significantly myopic (Shen, Vijayan et al. 2005). Interestingly, the elongation occurs in both the vitreous and the (optically null) anterior chamber. Axial length growth can also be slowed with positive lenses (Shen and Sivak 2007).

Therefore there are potential mechanisms which allow for focal regulation in a marine camera-type eye at the level of the lens, retina, or a combination of both. While regulation of lens and
retinal growth in the vertebrate fish eye in no way implicates a common modality in a cephalopod eye, taken in light of the high degree of convergent evolution between the species, it demonstrates the possibility that both components may be able to regulate emmetropisation in a co-ordinated manner.

7.3. Accommodation as an alternative to emmetropisation

In general, the results presented do not support the idea that accommodation negates the need for emmetropisation in the squid eye. To respond to the lower focal power required to focus light in the blue spectral environment, the effective power of the lens could be decreased by moving laterally off axis, or anteriorly, away from the retina. Squid from the blue tank during Experiment 1 had more posteriorly displaced lens centres than squid from the orange tank (+0.11 ± 0.19mm versus -0.06 ± 0.19mm, \( p = 0.029 \)). While the measurements of lens decentration were not made under cycloplegia, the dark room and exclusive use of infra-red light prevented a visual stimulus for accommodation. Therefore, this difference in lens position between squid from the blue and orange tanks may represent a difference in tonic accommodation levels. In Experiment 3, the WOB cohort had anteriorly displaced lenses at 30 days post-hatching compared to both OBO and BBB cohorts, however the OBO and BBB cohorts, which were in the orange and blue tanks at 30 days post-hatching, respectively, were not significantly different from each other. Caution must be applied when discussing differences with the WOB cohort, as up until day 30 post-hatching (when the significant difference was measured) the squid had experienced a different visual environment (unfiltered tank), warmer temperature, and were significantly larger. The same absolute displacement of a larger, less powerful lens would have less of an effect on refraction, so it is difficult to attribute the displacement of the lens in the WOB cohort to the effect of the different spectral environment alone. At no other point was a significant difference seen in lens position between squid in the blue or orange tanks. This may be explained by the different time periods in each experiment. For Experiment 1, half of the squid were moved into the orange tank and left for 30 days, whereas for Experiment 2 and 3, the effect of changing the tank was assessed at a maximum of 5 and 15 days, respectively. A possibility exists that sustained changes in accommodation, caused by a permanent change in the spectral environment, manifests as a shift in tonic lens
position with eye growth, and that this effect is first observable between 15 and 30 days after the change in focal plane. Further support for this comes by comparing the lens centre of the OBO cohort from Experiment 3 at 60 days post-hatching, with the squid of the same age in the orange tank in Experiment 1. The mean lens displacement of the lenses from the Experiment 1 squid was more anteriorly displaced that the cohort from Experiment 3, however the 95% confidence intervals of the two cohorts of squid showed significant overlap (Experiment 3 OBO $Y_1$: 0.001 ± 0.130mm (95% CI -0.314 to 0.259), Experiment 1 Orange $Y_1$: -0.585 ± 0.191mm (95% CI -0.195 to 0.078mm)). Perhaps given more time in the orange tank, the lens centre in the OBO would move anteriorly.

However, the measurement of the centre of the lens in squid from the blue tank during Experiment 1 was the only time when the lens centre was significantly different from the centre of the retina circle (0,0) during any of the three Experiments (+0.111 ± 0.119, $p = 0.016$). This may indicate that this measure of lens centre in the squid from the blue tank is abnormally posterior, and the difference between the orange and blue is not due to anterior movement of the lens, but rather abnormal posterior displacement of the lens in squid eyes from the blue tank.

At no time was horizontal lens displacement ($X_L$) significantly different from the centre of the retina, and the variation in $X_L$ was less than anterio-posterior displacement ($Y_L$). Fish, like squid, have a spherical lens, which can move to change the effective power of the eye. In cartilaginous fish, including elasmobranchii such as sharks, the resting state of the crystalline lens creates a myopic eye (Sivak 1990), and contraction of the protractor lentis muscle moves the crystalline lens anteriorly to see clearly in the distance (Sivak and Gilbert 1976). As the traditional definition of emmetropia describes a situation where the retina is aligned with the distance focal plane when the accommodative system is at rest, it raises the question of whether these vertebrates possess or even require emmetropisation, or perhaps the traditional definition is insufficient. In teleost fish, accommodation occurs after contraction of a retractor lentis muscle, which moves the lens temporally and posteriorly. It has been proposed that the temporal movement helps ensure the best optical quality over the highest retinal photoreceptor density, which is in the temporal retina. Squid lack a true fovea, but have a horizontal band of higher photoreceptor density, with no apparent nasal-temporal difference in density (Talbot and Marshall 2011). Therefore it would seem most likely that the lens would move along the anterior-posterior...
(optical) axis. This is supported by electrical and drug stimulation of the ciliary muscle which caused movement along the anterior-posterior axis in an octopus and cuttlefish eye (Beer 1897; Hess 1909). During anaesthesia with MS 222, squid lenses demonstrated movement in both directions along the anterior-posterior axis (Sivak 1982), however this may not represent lens movement in the alert animal, and does not provide information to the resting state of the lens. However, infrared photorefractio j of the cuttlefish eye failed to find a change in refraction along the anterior-posterior axis refraction during accommodation, but did find an increase in power along the medial-temporal axis, leading the authors to conclude that the lens may move along medial-temporal axis similar to teleost fish (Schaefefel, Murphy et al. 1999).

It should be noted that the displacement of the lens centre relative to the retina centre while in the dark is not a measure of accommodation, and it is possible that the relative anterior movement of the lens is part of the normal emmetropisation process; distinct from the short term change in accommodation. Additionally, it is possible that the accommodative system remains active in the squid whilst in the dark, as the eye may vary the focal length to search for bioluminescence in the deep ocean, which can indicate either prey, friend, or predator at night (Warrant and Locket 2004; Rader and Nyholm 2012). The lens could also move in the dark as a reflexive protective mechanism. While contained in the holding cell, there may be movement of the lens in response to touching the side of the holding tank. These concerns could be addressed by repeating the measures of lens decentration in cyclopleged squid eyes. How cycloplegia could be achieved has not been investigated, however anaesthesia with magnesium chloride caused a small increase in hyperopia in squid, measured by retinoscopy (Sivak, West et al. 1994).

7.4. Photorefrac tio

Photorefrac tion was conducted during Experiment 3 on squid aged 45 and 60 days post-hatching. As infrared light was used, measures of refraction do not represent the absolute refractive error of the squid in the tanks. However, as long as LCA remains constant, then relative measures of refractive error can be obtained. In general, photorefrac tion supports the concept that the regulation of squid eye growth seen with the changes in MR is to reduce refractive error. The cohort moved from the blue tank to the orange tank (OBO) showed a
significant decrease in refraction (+17.16 ± 2.36 to +11.38 ± 1.66, p = 0.007), while those moved from the orange to the blue tank (WOB) failed to show a significant change in refraction (+13.25 ± 1.96 to +16.04 ± 2.76, p = 0.149). This may indicate a level of asymmetry in the refractive response, with the response to the imposed myopic defocus being weaker than the response to simulated hyperopic defocus. However, the control group (BBB), which remained in the blue tank also showed a significant decrease in hyperopia over the same time period (+19.40 ± 1.75 to +14.74 ± 1.66, p = 0.005). This demonstrates a decrease in refractive error with increasing age, which would have reduced the required change in refraction from the OBO cohort.

While limited refractive data of cephalopods exists, previous measures of refractive error in cephalopods have also found hyperopic refractions. When refraction is measured with retinoscopy, the incandescent light source tends to give refractions towards the red focal plane (Nuboer, Bos et al. 1979). Refractive errors between -2.00 to +6.50D (mean +1.75 ± 2.4D), determined by retinoscopy, were seen in squid of unknown age, but whose overall lengths were between 26 to 36 centimetres (Sivak 1982); much larger than squid used for Experiment 3. Infrared photorefraction, which measures further beyond red than retinoscopy, has shown low hyperopia (reported as +2 to +3D) in three cuttlefish (Schaeffel, Murphy et al. 1999). Our measures of refractive error were significantly more hyperopic than previously reported, and there are a couple of factors that may contribute to this. As our squid were much smaller than animals used in other reports, the optical power of the eye would be much greater due to the inverse relationship between radius of curvature and optical power (Atchison and Smith 2000). Therefore for an equal misalignment of the focal plane and retina in a large and small eye, the refractive error would be more hyperopic in a smaller eye. The decrease in refraction seen in Cohort BBB during Experiment 3 (+19.40 to +14.74D), during which time the mean retinal diameter increased from 3.67 to 4.93mm, would support this. The small eye artefact describes the difference between the photoreceptor outer segments (where light is detected in the eye), and the reflective layer from which the refraction is determined during retinoscopy or photoretinoscopy. In small vertebrate eyes, the thickness of the retina can create a significant axial difference in distance between the reflecting layer (which is unknown (Mutti, Ver Hoeve et al. 1997)) from which refractive measures are made from, and the photoreceptor outer segments where light detection takes place, leading to a hyperopically shifted estimation of refraction. In cephalopods, this effect may be present, however it is likely smaller. Unlike the
vertebrate retina, the non-inverted cephalopod retina has the outer segments adjacent to the inner limiting membrane (ILM). If the ILM were the source of the observed retinoscopy reflex, it would tend to create only slightly hyperopic refractions. There is also an additional limiting membrane between the inner and outer photoreceptor segments, which would tend to create only slightly myopic refractions. Experiment 2 showed that the outer segments constitute almost 40% of the total retinal thickness, but it is not known if photons elicit an equal response along the entire outer segment. The measured hyperopic refraction could be explained by the use of infrared light (λ = 940nm) in the photorefractor, which due to LCA, would cause hyperopic refractions relative to focused white light.

The crystalline lens of some species is multifocal, and photorefraction has been used as tool to detect the presence of a multifocal lens. If present, multiple concentric rings are seen in the reflected light profile within the pupil, each circle correlating to a focal zone (Gustafsson, Ekström et al. 2012). However, no such rings were observed in any photorefractor images of *S. australis* or *O. tetricus* (casually observed). The cephalopod retina contains only a single opsin, therefore the absence of a multifocal lens is not surprising as the multiple focal zones can be used to bring different chromatic planes into focus (Gagnon, Söderberg et al. 2012).

In cuttlefish and squid eyes, pupil dilation is not-yoked, and is highly variable in speed (Douglas, Williamson et al. 2005; McCormick and Cohen 2012). However, photorefraction of the cuttlefish eye while hunting showed simultaneous and equal accommodation (Schaeffel, Murphy et al. 1999). While pupil responses were not directly assessed in our study, all photorefractor images showed fully dilated, circular pupils, and the high variability observed in *S. officinalis* was not seen in *S. australis*.

### 7.5. Growth of *Sepioteuthis australis* in a constrained environment

Squid grew throughout the experimental period at an average rate of 0.31mm per day. When measures of dorsal mantle length from Experiments 1, 2, and 3 are combined, squid growth can be seen to be linear up until 95 days post-hatching (Figure 7.1). Excluded from the regression are Cohort WOB, which were raised in warmer water for the first 30 days, and were significantly larger than the other squid at comparable post-hatch ages. The only other report of captive
rearing of *S. australis* (Hunt, Steer et al. 2011) does not include growth rate, however their oldest squid DML measured 16.5mm at 40 days post-hatching, and the second eldest 12mm at 31 days post-hatching. This data appears very similar to the size predicted by our linear regression.

![Dorsal mantle length measurements of all squid during Experiments 1, 2, and 3. A linear regression of growth of squid from both tanks showed the squid growth was linear (Pearson, n = 271, R² = 0.88) up to 95 days post-hatching, and dorsal mantle length (DML) increased by 0.31mm per day. Cohort WOB squid are excluded as they were initially raised in a different environment, changing their growth rate.](image)

*Sepioteuthis australis* hatchlings are the largest of the small-egg loliginoid family (Steer, Moltschaniwskyj et al. 2003), but hatchling size can vary significantly, even within the same egg mass. Steer et al. found a range from 4.33mm to 7.33mm in wild caught day one hatchlings (Steer, Pecl et al. 2003), while Bozzano et al. reported a range of 3.1mm to 5.0mm in his wild developed but lab hatched *S. australis* (Bozzano, Pankhurst et al. 2009). Hatchling size can vary due to different geographic regions, even at the same time of year and temperature (Moltschaniwskyj and Pecl 2007; Steer and Moltschaniwskyj 2007).
Males grow faster than females, and reach sexual maturity at approximately 150 days, and females just over a month later at 190 days (Triantafillos 2004). By adulthood, the higher growth rate results in males being slightly larger than females by between 1-20% (Triantafillos 2004). Body weight increases until spawning, by between 4-5% per day. Mantle length increases as a linear percentage (giving an exponential absolute length graph) throughout their life span, which can be up to 280 days (Triantafillos 2001; Pecl, Moltschaniwskyj et al. 2004).

Uncommon in the animal kingdom but not unusual in cephalopods, this growth continues past sexual maturity and until senesce (Boggs 1997). The largest dorsal mantle length measurement of S. australis was made in 1998 of 550mm (Wadley and Dunning 1998).

The squid used for the current study appeared to grow at rates similar to previous reports. Therefore, the housing of the squid was unlikely to have a significant effect on their overall growth, which may in turn have an effect on eye size. Additionally, as the growth rate is linear, it is more difficult to explain the lesser response in terms of the change in MR seen in older (90+ days post-hatching) squid compared to younger squid (30-60 days post hatching).

### 7.6. Limitations of the current study

#### 7.6.1. Ocular biometry

The technique used to obtain retinal diameter was infrared photography of live squid, viewed through the largely transparent squid body. A circle was fitted to a series of points marked on the outer edge of the dark retinal cup. In larger squid, the temporal aspect of the eye cup became obscured by the optic lobe as it increased in thickness. This may have affected the determination of the circle size, especially if the eye cup was not well represented by a circle, although this error would have likely been equal between the squid from the two spectral environments. Additionally, the points used for fitting the circle were marked on the outside of the eye cup; the true focal plane of the eye at the outer-segment of the photoreceptors would have a slightly smaller radius. Fortunately, the cephalopod sclera is thin, as is the retina, as it only contains photoreceptors. Measuring the distance from the outside of the sclera to the proximal edge of the outer segment of photoreceptor on day 70 post-hatching squid showed
the total thickness of the eye cup to be approximately 150µm when the measured retinal radius was 3.0mm. This would cause an overestimation of the focal plane of approximately 5%.

However, Experiment 2 showed there was no significant difference in photoreceptor length in eyes of squid from the blue or orange spectral environments. If the thickness of either the choroid or sclera changed, the plane of the photoreceptors would be altered and would remain undetected with this technique. However, if there was a difference in choroidal or scleral thickness between squid from the blue or orange tank, it would only constitute a small difference relative to the measured lens size, as the choroid and sclera only comprise approximately 35% of the total thickness of the eye cup wall.

Due to the overestimation of the retinal diameter, but not the lens diameter, which could be accurately aligned, MR would also tend to be overestimated.

7.6.2. Degree of change

Squid LCA was not directly measured as part of this thesis, so the observed changes cannot be correlated with those expected. Using coloured filters in front of a retinoscope, the measured dioptric effect of LCA in Illex illecebrosus (Short finned squid) was -3.50D from red (\(\lambda = 656\text{nm}\)) to blue (\(\lambda = 496\text{mm}\)) in large specimens (Sivak 1982). This value is quite high, possibly as the effect of LCA on vision is reduced in cephalopods due to the single opsin retina (Land 2002), which allows some of the visual spectrum to remain undetected, or the wavelength restricting effect of the ocean (Sivak 1982). However, LCA is not a dioptric value, but rather a variation in refractive index with wavelength. In humans, for example, LCA in adults is approximately 2 dioptres, however in infants, LCA is 1.7x greater due to the smaller, more powerful eye (Wang, Candy et al. 2008). In this respect, it is more appropriate to discuss LCA in terms of percentage difference in refractive power between wavelengths. Between 450-700nm, the focal length of the octopus lens varies by approximately 4% (Jagger and Sands 1999), which is not too dissimilar to fish lenses (Jagger and Sands 1996). Therefore, the estimated difference in focal planes created by the spectral filters is 1.76% between the two spectral environments (based on 4% LCA over 250nm spectral range in octopus (Jagger and Sands 1999)). At 60 days post-hatching, refraction between squid from WOB and OBO cohorts differed by +4.66D, which would infer an lens power of approximately +260D for squid in the blue tank.
One factor that was not fully controlled between the tanks was luminance. In vertebrates, increased luminance can influence eye growth (Cohen, Belkin et al. 2011), and may protect against the axial elongation seen in myopia (Ashby and Schaeffel 2010). The squid in the blue spectral environment were exposed to approximately 1.16x the luminance of squid in the orange tank. The influence of luminance on cephalopod eye growth has not been investigated, however it has in *Siganus rivulatus* (Marbled spinefoot), a Rabbitfish traditionally found in the Red Sea, which has recently migrated through the Suez Canal to colonise the Mediterranean Sea. Compared to the native Red Sea, the Mediterranean Sea is dimmer at all depths. While comparing MR of fish in the brighter Red Sea to that of the duller Mediterranean, it was found that fish eyes adapted to the dimmer environment by decreasing MR (decreasing the f-stop), in order to increase the light gathering ability of the eye (Gagnon, Shashar et al. 2011). Therefore if squid were to follow a similar pattern, the squid in the dimmer orange would be expected to demonstrate a smaller MR: the opposite of what was seen. This suggests that the effect was either not present, too small in relation to the focal plane changes to be observed, or the squid eye is unable to adapt to a permanent change in luminance levels. Either way, if tank luminance had an effect on the results, it would have done so in a manner expected to reduce the difference in MR between the tanks.

### 7.7. Significance of emmetropisation in squid

That squid possess emmetropisation, while a new finding, is not surprising. Squid are visual predators with large, high acuity eyes which grow throughout their life. While hard to appreciate due to the similar superficial appearance of the marine vertebrate and cephalopod eye, the last common ancestor possessed no more than a pit type eye (Fernald 2004; Parker 2011). Since it lacked a lens (Land 2012), this common ancestor was therefore unlikely to have a need for emmetropisation. Thus, alongside the evolution of camera-type eyes, emmetropisation appears to have evolved at least twice, in both vertebrates and cephalopods.
Chapter 8. Methods: Assessment of cephalopod visual function

8.1. Measurement of *Octopus tetricus* visual acuity

8.1.1. Experimental subjects

All experiment procedures were approved by the Animal Ethics Committee of The University of Auckland, and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Visual acuity was measured in a total of 3 octopuses. In addition, accommodative function was measured in one octopus. Two octopuses were captured opportunistically from the Pacific coast at Leigh, Auckland, New Zealand (approximately 36.31S, 174.78E) by local fishermen using crayfish pots, which the octopuses chose to occupy (Brock, Saunders et al. 2006). One further octopus was captured from an oyster farm at Whangaroa in the Far North region of New Zealand (35.05972S, 173.74002E), and was transported back to Auckland by road, with frequent stops to refresh the water.

Upon arrival at the University animal facility at Grafton, the water in the transporting bucket was slowly diluted (approximately 15% exchange every 5 minutes) with water from the aquarium to allow gradual acclimatisation. After a minimum of 25 minutes, the contents of the bucket, including the octopus, was poured into an individual tank (Figure 8.1), and the octopus was allowed to establish itself in the new environment. Acclimatisation was determined by a lack of red skin colouration (indicative of stress) and the acceptance of food, and generally took less than 24 hours. Some animals took a while to familiarise themselves with the transparent glass walls of the tank, and would jet themselves into the sides of the tanks. However, this behaviour soon subsided, and they almost always took up residence in one of the terracotta flower pots provided for their den.
8.1.2. Octopus tank

The octopuses were kept in a 940 litre closed salt water aquarium comprising a 400 litre communal sump and 3 purpose built, 180 litre tanks. The individual tanks were custom built from 10mm glass with removable lids, and measured 800h x 550d x 400w millimetres (Figure 8.1). Each octopus was housed individually to prevent cannibalism, and dividers were placed between the sides of the tanks so that each octopus could not see the next-door tank. The front and back of the tank was not covered so the octopus could see into the room, with a maximum viewing distance of approximately 0.5 meters through the back of the tank, and 3.0 metres though the front of the tank. Octopuses acclimatised to the tank for up to a week before experiments began. Further details of the aquarium arrangement and octopus maintenance are provided in Appendix 1.

Figure 8.1 - Individual holding tank design showing relative positions of inflow, outflow slit, and overflow box. Water was forcibly pumped in through the larger opening on the side, which sits just above the water line. It hits the far side of the tank, stirring up the tank water with bubbles. The slit overflow was covered with a mesh filter when occupied. The two outflows provided greater than twice the drainage capacity of the inflow rate to prevent flooding should the octopus block one. Infrared water level sensors were placed above the waterline near the overflow. If the line of sight was broken, inflow ceased until the water level returned to safe levels. Not shown is the glass lid, which was weighted down with bricks.
8.1.3. Lighting

Average illumination at tank level was 200 lux (range between tanks of 190 to 210), provided by ceiling mounted fluorescent tubes (Starcoat T5/f28W, General Electric, USA). Additional spot lighting was provided with compact fluorescent tube desk lamps through a diffuser above the tanks while the experiments were running, which permitted higher quality images (lower gain) on the video camera.

8.1.4. The fixation reflex

During routine maintenance, the octopuses were observed responding to new or interesting visual stimuli with a physical response, which will be referred to as the ‘fixation reflex’ (Figure 8.2). It is distinct from the previously described ‘dymantic display’ (Wells 1978), (extreme darkening of the skin around the eyes in a ring like pattern), as it is much more subtle, transient, and frequent.

![Figure 8.2 - The fixation reflex. Six snapshots of an octopus eye over a two second period during stimulus presentation (inset – top left). a) The octopus at rest, with an isoluminant grey display. b) Once the stimulus is presented, there is firing of chromatophores along the superior margin of the pupil. c) The pupil widens, chromatophores along the inferior pupil margin darken, and the skin around the eye begins to contract. A hemispherical bulge under the iris, presumably created by the anterior movement of the crystalline lens, is apparent. d) Maximum darkening has occurred, which now extends to the ‘faux pupil’ on the left hand side of the eye. e) Once the stimulus has been removed, the skin around the eye relaxes, and f) the pigmentation around the pupil returns to resting levels. As a dynamic reflex, the effect was more apparent as a ‘brown flash’ in video footage. Video can be seen at: http://youtu.be/irNvTozRmvM.](http://youtu.be/irNvTozRmvM)

When a new object of interest was observed by an octopus, chromatophores in the iris and surrounding ‘eyelid’ tissue momentarily contracted, causing a darkening of the areas that are a
continuation of the horizontal pupil. At times, the response was more exaggerated, and pupil size increased with a small saccadic movement; at the lower level, there was a mild darkening of the superior iris alongside the pupil margin. An increase in pigmentation has been attributed to neurological firing of the radially arranged muscles in the chromatophores (Messenger 2001). The effect was fleeting, and the appearance returned to baseline within two seconds. A possible mechanism could be movement of the lens while accommodating to fixate an object, which may mechanically stimulate the chromatophores surrounding the eye, and partially dilate the iris.

8.1.5. Acuity test setup

The fixation reflex was monitored unobtrusively by a tripod-mounted camera (Panasonic WV-CP654E, Japan) with a 55mm f/1.2 lens (1:1.2/12.5-75, CBC Computar, USA) on two 20mm focal extenders, focused on the forward facing eye. The camera output was analysed and recorded in real time using the live capture feature in Windows Movie Maker (version 6.0, Microsoft, USA). The testing distance was approximately 1 meter; variations existed due to animal position and room setup limitations, but the program was calibrated to the exact distance between the eye and stimulus for each experiment. If the animal moved during testing, the testing was paused until the animal resettled and the camera was repositioned and refocused, and the psychophysics program was recalibrated for the new distance. During acuity testing, the sides of the octopus tank were covered with black card, and a wooden tunnel was made between the front of the tank and the screen on which the stimulus was presented. This prevented distractions and confounding movements during the observations. Unfortunately, movement of suspended debris inside the tank water, particularly shed octopus sucker lining, occasionally diverted attention and induced a false positive response. While these events happened infrequently, the recording of acuity was set to require two consecutive ‘stimulus seen’ responses in order to reduce this distraction bias.

8.1.6. Stimulus creation and presentation

Acuity testing was conducted through a custom software program written in GNU Octave (version 3.2.0, obtained from www.gnu.org/software/octave), running the Psychtoolbox module (version PTB-3, obtained from http://psychtoolbox.org, see Figure 8.3). A vertically oriented sinusoidal grating with overlying Gaussian blur transparency (Gabor patch) was displayed on an
isoluminant grey background, and shown full screen on a 19” LCD monitor (E196FPF, Dell, USA). The Gaussian transparency was set so the stimulus was always centred on the screen, with no cut-off from the edges of the monitor. The monitor had a Michelson contrast of 98%, 300 cd/m brightness, native resolution of 1280x1024 at 75Hz refresh rate, and pixel dot pitch of 0.294mm.

Figure 8.3 - Algorithm for demining the highest cycles per degree (CPD) required for eliciting the fixation response. i = index for counting the ten separate iterations per day, SI = Stimulus index, counts the total number of presentations, NC = Noise check, requires two consecutive positive responses to minimise the false positive rate caused by non-stimulus generated fixation responses.

A method of limits from non-seeing to seeing using a 15% staircase was chosen to minimise the number of seen presentations, so as to reduce the opportunity for habituation. The program showed Gabor patches with decreasing cycles per degree until two consecutive positive responses were noted. To reduce errors of expectation from the observer, the starting frequency was randomly selected from a range well outside the expected maximum visual acuity, and not revealed until after the experiment had finished. The Gabor patch was presented and alternated with an identical grating offset by π at 5Hz which gave the impression of a flickering stimulus.
The flickering stimulus was presented for two seconds, then a two second break, then again for two seconds. The flicker was presented twice to increase the likelihood of being observed by the octopus at rest. Between presentations, the monitor displayed the background uniform isoluminant grey.

The testing was repeated 10 times in each session, for a total of 5 sessions (50 trials). The output was a sample mean and standard error for each octopus.

The validity of the response detection was first verified in a pilot study, which differed in that it had a microphone feed into the live video and was recorded. The microphone was used to record ancillary test notations, such as pausing or recalibrating the test if the octopus moved. Two independent observers watched the video looking for positive responses. Similar positive responses were noted, and equal final visual acuity was computed by each observer, suggesting that the ‘double positive’ response required for the acuity measurement improved test repeatability, and that the test was reliable.

8.1.7. Assessment of accommodation

Accommodation was assessed using Nott dynamic retinoscopy. This technique involves using an accommodative stimulus at a known distance, and moving the retinoscope (and observer) away from the stimulus until a neutral reflex was seen (Manny, Chandler et al. 2009). At this point, the focal plane of the eye was congruous with the retinoscope position, and by measuring the distance between the retinoscope and the stimulus, the error of accommodation could be established.

The testing was performed in a dark room, using an internally illuminated children’s toy as the accommodative stimulus. The stimulus, about 40mm across, varied in brightness and cycled between different hues. It contained both high and low frequency details, from the gross outline, to small printed details. It was manually jittered during retinoscopy in order to maintain fixation. Retinoscopy was performed along the horizontal meridian, as due to the iris shape, this provided the longest stroke length. Five measures were made at 0.25m, 0.5m, 1m, and 1.5m, performed in a randomised order by two experienced retinoscopists. A mean of both retinoscopist’s measurements at each distance (10 measurements per distance) was recorded. No significant astigmatism was noted at any time.
The measurement to correct the accommodative error was made in air, while the octopus eye was in water viewed through a glass tank, which exaggerated the vergence of light. To correct for this, calculated accommodative errors were divided by 1.34 (refractive index of salt water) to provide the error at the corneal plane under water, as is standard practice for retinoscopy of aquatic animals (Hueter and Gruber 1980).

8.1.8. Age determination of *Octopus tetricus* by stylet analysis

While octopus lack the shell common in the Mollusc phylum, small internal remnants (stylets) remain as a paired, chitinous, elongated structures on the inside of the dorsolateral wall of the mantle hood, adjacent to the gills (Doubleday, Belton et al. 2008). In microscopic cross-section, stylets consist of series of concentric bands, and it is believed that these bands accumulate daily (Leporati, Semmens et al. 2008). These bands can provide a method for determining the approximate age of the octopus. In some species, it is difficult to distinguish the pre-hatch nucleus from the initiation of daily deposition (Barratt and Allcock 2010). Additionally, there may be variation in the deposition rate between species (Doubleday, White et al. 2011). However, as all three of our octopuses were of the same species, counting stylet bands should allow relative age, if not absolute, determination of age.

Stylets were removed from three octopuses post-mortem and stored whole in 70% ethanol prior to analysis. To prepare for ultramicrotome sectioning, a one millimetre thick segment was taken just below the stylet elbow (Leporati, Semmens et al. 2008), rinsed in 0.1M phosphate buffered saline, and dehydrated in increasingly concentrated ethanol (70%, 80%, 90%, and 100% twice) for 10 minutes, before being rinsed twice in 100% acetone for 15 minutes. The samples were put into a 3:1 mix of resin and acetone and left overnight. The following day, the sample was moved into 100% resin for one hour, then fresh resin for another 5 hours. The tissue samples were aligned in a bullet mould with fresh resin with a paper label identifying the octopus, and allowed to polymerise at 60°C for 24 hours.

The resin bullets were sectioned at 150 microns on an ultramicrotome (EM UC6, Leica, Germany). The sectioned tissue was mounted on a slide with water, and immediately viewed in dark field illumination, and photographed at 200x magnification (Camera: DC500, Leica, Germany on microscope: DMRA2, Leica, Germany). The images (Figure 8.4) were opened, aligned, and joined in ImageJ.
Figure 8.4 - Example of a transverse section of an octopus stylet after embedding in resin. The nucleus can be seen as the dark area towards the bottom centre of the image. The parallel striae are an artefact of the sectioning, however the circumferential rings are visible even without additional magnification.

Alternating radial stripes of high and low contrast rings were counted, with each light and dark pair representing one day. Ideally the count would be along a continuous straight radial, however, due to sectioning artefacts or areas of poor contrast, this was not always possible. In these cases the circumferential band was followed until a higher quality area was reached, and then counting could resume along this new partial radial. The bands were marked using the cell count feature of ImageJ to prevent errors of duplication, and to provide a mechanism for auditing.
Two distinct counts were performed by two experimenters, with a mean count taken as the octopus age in days, at time of death. This count was then used to retrospectively age the octopus, and calculate its age when tested.

8.2. Measurement of squid *Sepioteuthis australis* modulation transfer function

8.2.1. MTF target preparation

A subset (see Figure 8.5) of the USAF 1951 test pattern vector image was printed onto photographic paper (HP Vivid Photo Paper, Hewlett-Packard Company, USA).

![USAF 1951 Test Pattern Target (Group, Element)](image)

Figure 8.5 - The range and increments of USAF 1951 MTF (inset) elements viewed through the squid lens in this experimental setup. The lower limit was restricted by the field of view of the lens and the upper limit was limited by the ink bleed of the photographic paper at 12.57 cycles per degree.

When viewed through a 7x magnifying loupe (Peak #1975, Peak Optics, United States), the maximum resolvable resolution by the human observer’s eye was Group 5, element 2, corresponding to 36 lines/mm on the paper. Higher magnifications on two different
microscopes revealed this limit was due to bleeding of the photographic ink (creating a black spot rather than alternating black and white stripes), rather than the spatial resolution limit of either the loupe or the observer’s eye. In our setup, that translated to a maximum angular resolution of 12.57 cycles per degree.

Multiple squid crystalline lens holders were prepared by drilling a hole through the centre of a concave contact lens case, with a range of diameters from 3 to 6mm in 0.5mm increments. A holder was selected so that the ciliary band around the lens equator would prevent the lens from falling through the holder – if an exact fit could not be found, the next smallest hole size was selected. The holder, with squid lens in place, was immersed in a small tank of salt water, so that the ciliary band (approximate equator of the lens) was positioned 20mm above the target image, with the 7x loupe placed on top of the lens holder (Figure 8.6). The water level in the tank was at the level of the ciliary band. Prior to viewing, an additional drop of water was added to the anterior lens surface to provide a smooth optical surface. This prevented an exaggerated effect of small surface imperfections due to the air : lens interface, rather than the in vivo aqueous : lens interface. Looking down through the loupe showed the magnified target focused through the squid lens. If required, focal adjustments could be made to the eyepiece of the loupe but this was rarely necessary. Occasionally, the target image required translation to ensure the smallest details were being observed through the centre of the lens. This could be achieved by moving the target image, rather than moving the optical setup.

The maximum resolution of a lens when viewing the test pattern in this way is given by the smallest resolvable line pair, defined as seeing at least two (of six) distinct black bars (Sweeney, Haddock et al. 2007). The spatial resolution was calculated for any given set of bars using (Glynn 2002):

\[ Cycles \ per \ mm = 2^{-\left\lfloor \frac{Group + (Element - 1)}{6} \right\rfloor} \]

The inverse of cycles per mm gave the linear width of each cycle, and taking the arctangent of the linear width divided by the viewing distance gave the angular resolution in cycles per degree. As poor lens extraction technique or natural decay of the lens post-mortem would likely
decrease the optical quality, the highest MTF value of the two lenses from each squid was used for analysis.

![Image of USAF 1951 MTF test pattern through the lens of a squid from the orange tank. The dark ring is the ciliary band (marked with *); the transparent lens sits inside, with Group 2 and 3 imaged through the lens. Scale bar in top right is 1mm.]

Figure 8.6 - View of the USAF 1951 MTF test pattern through the lens of a squid from the orange tank. The dark ring is the ciliary band (marked with *); the transparent lens sits inside, with Group 2 and 3 imaged through the lens. Scale bar in top right is 1mm.

### 8.2.2. Experimental subjects and measurement protocol

Twelve 100 day old squid (6 from the orange tank, 6 from the blue tank, 5 days after Experiment 2 (see Chapter 5.2.2) were cold anaesthetised, decapitated, and their right eye enucleated while immersed in salt water. The whole eye was transferred into a larger vessel with fresh sea water to prevent contamination of the other eye, and then the lens and ciliary band were dissected out by snipping the lens zonules with dissection scissors. The lens (mean diameter approximately 4.0mm) was manipulated with tweezers gripping the ciliary band, so as to avoid contact with the lens surface. The lens was placed anterior side up in a prepared concave holder. The target pattern was identified through the lens, and the smallest distinguishable element pair (vertical and horizontal) was recorded. The lens was discarded, and the process was
repeated for the left eye. The time delay between measuring the right and left lens was not formally recorded, but was approximately five minutes, with the right eye always analysed first.

8.2.3. Statistical analysis

Statistical analysis was performed in SPSS (Version 20, IBM, USA). Paired t-test was used for paired eye correlation, while significance between tanks was tested with one way ANOVA.
Chapter 9. Results: Assessment of cephalopod visual function

9.1. Octopus visual acuity

Visual acuity measurements were obtained on three octopuses. The ages at which the measures were made were determined retrospectively from the stylet analysis. The visual acuity measurements for the three octopuses ranged from 11.13 ± 3.16 to 13.32 ± 6.31 cycles per degree (CPD, mean ± SD of 50 trials per octopus over 5 days), with a mean of 12.23 ± 1.61 CPD. The octopus age on the first day of testing ranged from 111 ± 15 to 284 ± 17 days. There was no correlation between age and acuity (Pearson, R = -0.299, n = 3, p = 0.807, Figure 9.1).

![Figure 9.1](image.png)

Figure 9.1 - Mean visual acuity results from three octopuses (CPD, cycles per degree) from 50 trials per octopus, versus age at the first day of visual acuity testing. Error bars are SEM.
9.2. Octopus accommodation

Accommodation was assessed in one octopus, with a mean of 10 measurements by two independent retinoscopists (5 measures per retinoscopist at each distance). In a darkened room while the octopus was eating, a mean refraction of $+1.54 \pm 0.30$ dioptres (D) was observed after adjusting for the working distance of 50 centimetres. When an accommodative target was placed at 1.5 metres (m) from the octopus, $0.63 \pm 0.02$ D of accommodation was measured, close to the exact accommodative demand of 0.67D. As the target moved closer, the octopus continued to accommodate, although to a lesser degree (i.e. the accommodative lag increased, Figure 9.2) so that only 2D of accommodation was seen with a 4D stimulus. No significant astigmatism was seen during testing, although this was not formally assessed. Additionally, accommodation was rapid and sustained while the target was illuminated.

![Accommodative response curve for a single Octopus tetricus.](image)

Figure 9.2 - Accommodative response curve for a single Octopus tetricus. Accommodation for targets greater than one metre was accurate, however an increasingly large accommodative lag developed when the target was moved nearer than one meter. Error bars are 95% confidence intervals, and are smaller than the marker for 2D and 4D data points.
9.3. Squid MTF

Measurement of maximum resolution (CPD) through the squid lens was obtained in a total of 12 squid (6 from the blue tank, 6 from the orange tank, Table 9.1).

<table>
<thead>
<tr>
<th>MTF</th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
<th>95% CI</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue</td>
<td>6</td>
<td>7.19</td>
<td>0.62</td>
<td>6.54 - 7.85</td>
<td>6.27</td>
<td>7.90</td>
</tr>
<tr>
<td>Orange</td>
<td>6</td>
<td>5.92</td>
<td>1.22</td>
<td>4.64 - 7.21</td>
<td>3.94</td>
<td>7.04</td>
</tr>
</tbody>
</table>

Table 9.1 - Analysis of maximum resolvable MTF in 6 squid from the blue and orange tanks. Units are cycles per degree (CPD).

The right and left eye values were highly correlated (Paired T-test, $R = 0.79, p = 0.002$, ), however where a difference between the eyes was observed, the right eye was higher in five out of six cases.
Figure 9.3 – Orthogonal regression of the maximum MTF for each squid between the right and left eye. There was a high degree of correlation between eyes ($r = 0.79$, $p = 0.002$), however the right eye, which was dissected and measured first, was higher 83% of the time. N = 12, however less points are visible due identical results.

Lenses taken from animals in both blue and orange tanks had normally distributed measures of MTF with equal variance between groups (Levene’s, $F = 0.582$, $p = 0.463$). The mean MTFs of squid lenses from the blue and orange tanks were not significantly different (ANOVA, $F_{(1,10)} = 2.56$, $p = 0.141$). The mean maximum MTF of all squid (n=12) was $6.56 \pm 1.14$ cpd.
Chapter 10. Discussion: Assessment of cephalopod visual function

The results presented in Chapter 9 suggest that both Sepioteuthis australis, and Octopus tetricus possess eyes with high visual acuity. Clearly, the attribute of high acuity does not itself prove the presence of an emmetropising mechanism, but it does suggest that the size of the cephalopod eye is matched to the power of the optical components. As discussed in Chapter 2, an unfocussed eye with the potential for high acuity would be disadvantaged in evolutionary terms and may be selected against. Furthermore, both octopus and particularly squid are predators from birth, suggesting a need for high acuity from an early age. Therefore, the finding of high visual acuity provides a strong rationale for investigating the presence of an emmetropising mechanism in cephalopods. Further rationale is provided by the rapid growth post-hatching, as the size of the cephalopod eye typically increases by an order of magnitude throughout development, suggesting a continual requirement for maintaining co-ordinated ocular growth. The presence of some form of emmetropisation mechanism in the cephalopod eye might therefore be expected, and the results of the LCA experiments (Chapters 6 & 7) in squid demonstrate that this is indeed the case.

10.1. Visual acuity of Octopus tetricus

The results show a mean distance visual acuity in adult Octopus tetricus of 12.23 ± 1.61 cycles per degree (CPD). Visual acuity has been previously reported in the octopus at between 7.5 to 12 CPD using behavioural methods, which require preconditioning of the octopus to attack a spatial grating (Muntz and Gwyther 1988). In their behavioural study, positive responses to a grating stimulus above threshold were obtained approximately 80% of the time, although one octopus was closer to 90% (Muntz and Gwyther 1988). This is the equivalent of a 10-20% ‘false negative’ rate. Our methodology, utilising a seemingly natural reflex, has an advantage over a trained behavioural response, as the fixation response requires less energy expenditure and less motivation from the octopus, and therefore less potential for a false negative. False negatives during the presentation of our stimulus would lead to overestimation of visual acuity, as the frequency of the Gabor patch was always decreased from non-seeing to seeing. However, our
methodology required a double-positive response (see Chapter 8.1.6). This gave the octopus a ‘second chance’ to detect the stimulus, which would likely reduce false negatives. Our pilot methodologies (not reported in this thesis) used a method of constant stimuli to present the Gabor patches, in an attempt to create a frequency response curve. However, during the many presentations of the repeated spatial frequencies, the octopus stopped displaying the fixation reflex, even to spatial frequencies much lower than their threshold determined from previous trials. Habituation is considered a primitive form of learning, and has been demonstrated in octopus during play (Mather and Anderson 1999; Kuba, Byrne et al. 2006), as well as after repeated visual presentations (Kuba, Zullo et al. 2006). Because of this apparent visual habituation, we were unable to record a repeatable threshold with the method of constant stimuli. However, by employing the descending staircase method, we found that the responses of the three octopuses were consistent, with no trend towards poorer VA over time: this suggests that no habituation took place.

As only descending staircase values (with no reversals) were used to determine the mean visual acuity, our method may have underestimated visual acuity (García-Pérez 1998). Another factor that may have caused an underestimation of visual acuity was the unknown accommodative state prior to testing. During the testing of visual acuity, the walls of the tanks were covered with plain black paper, and a tunnel was placed over the front of the tank. The stimulus screen was at the end of the tunnel, the most distant object in the horizontal visual field. Even when not displaying a stimulus, the backlight of the monitor would have been a ‘light at the end of the tunnel’, and may have been sufficient to provide an accommodative stimulus. Consideration was given to priming the octopus with a visual stimulus on the screen prior to each presentation. However, difficulties in distinguishing the fixation response to the testing stimulus from a prolonged response to the priming stimulus, in addition to the unknown speed of the accommodative system, led to dismissal of this idea. The consistency between octopuses (the range between highest and lowest octopus mean visual acuity was 2.10 CPD) suggests that accommodation, if not focused on the monitor, was at least reasonably constant between animals.

Previous studies have shown that octopus may be able to discriminate vertically orientated spatial gratings better than horizontally orientated gratings, although the reason for this is not understood (Muntz and Gwyther 1989). Discrimination of the width of rectangles is also
improved if the long axis is orientated vertically (Sutherland 1963). As the Gabor patch grating was always orientated vertically, this may give a higher estimate of octopus visual acuity than if a horizontal grating were to be used. A possible cause for this difference in acuity could be due to the horizontally rectangular shape of the pupil, which may act as a stenopaiic slit (Singh and Khanna 1967). A larger pupil provides a shallower depth of focus; therefore the rectangle would give different depths of focus along different meridians. If the eye was focused away from the screen as the stimulus was presented, a wider depth of focus would allow for the stimuli to be discriminated over a greater range of defocus.

Cephalopods, including both octopus and squid, are able to detect and discriminate between different polarisations of light (Shashar and Cronin 1996; Shashar, Rutledge et al. 1996; Saidel, Shashar et al. 2005). The ability to detect the polarisation of light probably results from the orthogonal anatomical organisation of rhabdomeres in the cephalopod retina (Moody and Parriss 1961; Saidel, Shashar et al. 2005). Polarisation may provide additional visual information beyond luminance contrast, as it could enhance contrast if two different objects reflect different polarisations of light (Mäthger, Shashar et al. 2009; Cartron, Dickel et al. 2013). Our stimulus presentation screen had a polarising filter, which transmitted light polarised at 45 degrees. While this would have decreased luminance, no additional polarisation information would have been provided, as the blank screen and all stimulus presentations were polarised identically.

Octopus eyes grow considerably from birth to senescence (Derusha, Forsythe et al. 1987), and if the total number of photoreceptors also increases, there is the potential for visual acuity to increase with age because of the increase in angular photoreceptor density (Pankhurst, Pankhurst et al. 1993). One initial aim of the experiments was to test this by measuring visual acuity over a range of octopus ages. Unfortunately, none of the octopuses were juvenile at capture, so their growth rate may have already slowed in their adult years (Cortez, González et al. 1999). However, there was no obvious trend of visual acuity over time in the three octopuses who had their acuity measured. While not measured, there was no appreciable increase in size of any of the octopuses during captivity, so changes in visual acuity due to angular photoreceptor density would not have been expected.
10.2. Optical quality of the *Sepioteuthis australis* lens

While behavioural measurements of visual acuity are considered the best representation of true visual function (Browman, Gordon et al. 1990), only one paper details behavioural measurement of visual function (the presence of colour vision) in a non-benthic, free-swimming cephalopod, the cuttlefish (Mäthger, Barbosa et al. 2006). However, estimates of visual acuity can also be obtained through anatomical measurements such as photoreceptor density (Matsuda, Torisawa et al. 2005), or lens optical quality (Artal, De Tejada et al. 1998). An estimate of the visual acuity of *S. australis* was therefore made indirectly, by determining the maximum level of detail that could be resolved through an excised crystalline lens. The MTF through the crystalline lens has been measured in eight different squid species (Sweeney, Haddock et al. 2007), which revealed a wide range of crystalline lens optical quality. The resolution at which 20% contrast between the black and white components of the MTF grating remained, ranged from 4 to 19 CPD. The highest MTF value obtained was over 22 CPD - the highest spatial frequency grating used in the study – and was obtained in *Vampyroteuthis* (Sweeney, Haddock et al. 2007). *V infernalis* is an ancient deep-sea squid, with features of both squid and octopus (Hoving and Robison 2012), and the most evolutionarily distant from *S. australis* (Lindgren, Giribet et al. 2004). The maximum MTF value obtained for *S. australis* in this study (6.56 CPD) fits within the previously reported range, but at the lower end of the spectrum. As the squid from which the lenses were taken were 70 days post-hatching, prior to sexual maturity (Pecl and Moltschaniwskyj 2006), it is possible that crystalline lens quality would continue to improve over time (Kröger 2013), as the composition of the squid lens changes significantly as the squid grows from juvenile to adult (Sivak, West et al. 1994). If visual acuity improves with age, it may permit progressive fine-tuning of the emmetropisation response.

MTF estimates can be considered conservative, as sacrifice of the animal and poor dissection technique will likely reduce optical quality (Sweeney, Haddock et al. 2007). However, if the technique is perfect, it is unlikely to underestimate optical quality, even if the retinal photoreceptor density suggests a higher resolution. This is because the crystalline lens will act as a low pass filter, and prevent high spatial frequency information from reaching the retina. Additionally, the retinal photoreceptor mosaic may be subject to neural convergence (which can improve temporal sensitivity), and therefore photoreceptor density alone can lead to significant overestimations of visual acuity (Lee and Bumsted O’Brien 2011).
10.3. Comparison of visual acuity in Animalia

Our recorded visual acuity of 12.23 CPD in *O. tetricus* and the maximum MTF resolution of 6.56 CPD in *S. australis* compares favourably to many animals (Table 10.1). Octopus visual acuity rivals most land predators and is significantly higher than marine vertebrates, alongside which they have directly evolved in the same visual environment.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Maximum visual acuity (CPD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wedge-tailed Eagle (<em>Aquila audax</em>)</td>
<td>140 (Reymond 1985)</td>
</tr>
<tr>
<td>Brown Falcon (<em>Falco berigura</em>)</td>
<td>73  (Reymond 1987)</td>
</tr>
<tr>
<td>Human (<em>Homo sapiens sapiens</em>)</td>
<td>60  (Campbell and Green 1965)</td>
</tr>
<tr>
<td>Homing Pigeon (<em>Columba livia domestica</em>)</td>
<td>16  (Blough 1971)</td>
</tr>
<tr>
<td><strong>Octopus (Octopus tetricus)</strong></td>
<td><strong>12</strong></td>
</tr>
<tr>
<td>Beagle dog (<em>Canis lupus familiaris</em>)</td>
<td>9.5  (Murphy, Mutti et al. 1997)</td>
</tr>
<tr>
<td><strong>Southern Calamari (Sepioteuthis australis)</strong></td>
<td><strong>6.6</strong> (Lens MTF value)</td>
</tr>
<tr>
<td>Cat (<em>Felis catus</em>)</td>
<td>6.0  (Mitchell, Kennie et al. 2009)</td>
</tr>
<tr>
<td>Barn Owl (<em>Tyto alba pratincola</em>)</td>
<td>4.0  (Harmening, Nikolay et al. 2009)</td>
</tr>
<tr>
<td>Grass Goby fish (<em>Zosterisessor ophiocephalus</em>)</td>
<td>4.0  (Ota, Francese et al. 1999)</td>
</tr>
<tr>
<td>Archerfish (<em>Toxotes chatareus</em>)</td>
<td>3.57 (Temple, Manietta et al. 2013)</td>
</tr>
<tr>
<td>Guinea pig (<em>Cavia porcellus</em>)</td>
<td>2.7  (Buttery, Hinrichsen et al. 1991)</td>
</tr>
<tr>
<td>Tree shrew (<em>Anathana ellioti</em>)</td>
<td>2.4  (Petry, Fox et al. 1984)</td>
</tr>
<tr>
<td>Rhesus macaque (<em>Macaca mulatta</em>)</td>
<td>1.5  (Cowey and Ellis 1967)</td>
</tr>
<tr>
<td>Chicken (<em>Gallus gallus domesticus</em>)</td>
<td>1.5  (Over and Moore 1981)</td>
</tr>
<tr>
<td>Marmoset (<em>Callithrix jacchus</em>)</td>
<td>1.0  (Ordy and Samorajski 1968)</td>
</tr>
<tr>
<td>White Crappie fish (<em>Pomoxis annularis</em>)</td>
<td>0.13 (Browman, Gordon et al. 1990)</td>
</tr>
<tr>
<td>Common Seahorse (<em>Hippocampus taeniopterus</em>)</td>
<td>0.09 (Lee and Bumsted O'Brien 2011)</td>
</tr>
</tbody>
</table>

Table 10.1 – Comparison of visual acuity measured with behavioural (as opposed to anatomical) techniques for a variety of terrestrial and avian predators, as well as existing animal models of emmetropisation, and fish, who share the same optical environment. The report for human CPD, while behavioural, has the advantage of pre-priming and verbal conditioning, which is harder to achieve with other animals. The value for Sepioteuthis is likely higher than would be obtained using behavioural methodology, but is included as an indicative value.

During captive maintenance in our laboratory, the octopuses appeared to recognize different handlers by responding differently to them (e.g. by climbing to the top of the tank for food with
the primary handler), a trait that has been previously described (Anderson, Mather et al. 2010). Octopuses often appeared to be ‘observing’ us as we were working in the aquarium room, and would move to the front of the tank when we were preparing a shellfish for feeding.

10.3.1. Accommodation

The results, although limited to one animal, present physiological evidence for natural accommodation in an octopus. However, octopus accommodation may be limited: when the accommodative stimulus was closer than one meter, the accommodative lag, or the insufficiency of the accommodative system to focus at the required distance, progressively increased with the accommodative demand. When the stimulus was at 25cm from the octopus eye, the accommodative system was focused at 50cm. However, this inaccuracy does not necessarily translate to poor visual function for the octopus. Most humans also demonstrate accommodative lag, which could potentially decrease visual acuity at near (Subbaram and Bullimore 2002; Charman 2008). However in humans, activation of the accommodative system also causes pupil miosis which may increase the depth of focus sufficiently to negate most of the accommodative error (López-Gil, Martin et al. 2013). In cephalopods, the accommodative and pupil responses are not paired (Schaeffel, Murphy et al. 1999; McCormick and Cohen 2012), so they may not gain the optical advantage of a miotic pupil, and it would be interesting to determine whether visual acuity is reduced at near. However, accommodative lag may not be of great functional importance to the octopus. Most manipulation of objects by the octopus was conducted with the tips of stretched arms, such as reaching out for food or investigating around corners (Budelmann 1996). After food was contacted, it was passed up the suckers on the arm (which are mostly locally controlled (Young 1971)) towards the mouth, which is on the inside of the cone formed by the webbing between the proximal third of the arms. As their laterally opposed eyes are on the opposite side of the web, the octopus mouth remains hidden from view, therefore food manipulation, including the opening of shells, is conducted without visual input. As such, there may be little demand for accurate near vision. Furthermore, the accommodative near point may be limited by anatomy: as accommodation would require anterior movement of the lens away from the retina (Jagger and Sands 1999), the circumferential muscles attached near the lens equator would only allow for a limited range of movement (Wells 1978). However, this presupposes that the octopus eye is focused for distance when the accommodative system is at rest, which may not be the case.
The only other report of accommodation in a cephalopod is from photoretinoscopy of a cuttlefish (Schaeffel, Murphy et al. 1999). When prey was introduced, the eyes were seen to converge, and then become myopic relative to the plane of the photorefractor, indicating an increase in the dioptric power of the eye (Schaeffel, Murphy et al. 1999). The accommodation, estimated at 5.00D, appeared as a short pulse occurring for a fraction of a second prior to attacking the food. This is different to the sustained accommodation that we observed in the octopus. Interestingly, in the study by Schaeffel, the refractive change was only seen from in front of the animal (i.e. looking towards the temporal retina), and no change in refraction was noted from the side of the animal (along the visual axis), whereas in the current study all refractive measurements were made from the side of the animal. This may indicate differences in the movement of the lens between cephalopod species; the cuttlefish lens movement may have a lateral component (relative to the eye's optical axis), like teleost fish (Khorramshahi, Schartau et al. 2008), whereas the octopus lens may move along the optical anterior-posterior axis, similar to elasmobranch fish (Litherland, Collin et al. 2009). Further research is required to determine both the resting state of the octopus eye, and the full range and mechanics of the accommodative system.
Chapter 11. Thesis conclusions

This thesis has demonstrated the presence of an emmetropisation process in the invertebrate squid, and satisfied the Thesis Aims presented in Chapter 4. As the camera-type eye of cephalopod has an evolutionary history that is separate and distinct from that of vertebrates, our results suggest that over the past 500 million years, the process of emmetropisation has independently evolved at least twice; in vertebrates and also in cephalopods. As discussed in Chapter 2, understanding emmetropisation is important, because the prevalence of ametropia, and its associated morbidities, is increasing. While our understanding of animal emmetropisation is beginning to translate into treatments for human ametropia, the mechanisms by which emmetropisation operates is obfuscated by the complexity of the vertebrate retina. Chapter 3 introduced the Cephalopod phylum, which includes both octopus and squid. The convergently evolved cephalopod eye is attractive for emmetropisation research because of the simple optics, rapid and continuous growth, and importantly, the presence of a photoreceptor only retina. Chapters 8, 9 and 10 described the methods and results which showed that squid and octopus have high visual acuity eyes, and at least in octopus, accommodation. The high level of visual acuity in cephalopods strongly suggests the presence of accurate focal alignment: the eye is a metabolically expensive organ, and ametropia would both waste energy and be detrimental to the hunting performance of the animal.

The primary finding of this thesis is the presence of emmetropisation in squid. The methodology used to test this is detailed in Chapter 5. Several experiments were undertaken to show that the squid eye can compensate for hyperopic and myopic defocus, created by modifying the spectral environment to exploit inherent longitudinal chromatic aberration. The regulation of eye growth is in the expected direction, and occurs by adjusting the relative size of the retina in relation to the crystalline lens: a relationship described by Matthiessen’s ratio. The results in Chapter 6 show this process can occur quickly and reversibly, and the ability to regulate eye growth to minimise refractive error is present in squid aged between 30 and 95 days post-hatching. The particulars of measuring the change in eye growth are discussed in more detail in Chapter 7.

Now that emmetropisation has been demonstrated in the squid, the details of how it operates can be investigated. The complexity of the vertebrate retina often leads to modelling
emmetropisation as an equally complex process (Schaeffel and Howland 1988; Hung and Ciuffreda 2000). The simplicity of the components in this cephalopod model suggest that emmetropisation in this species may not be so complex. This in turn should raise questions about how complex the vertebrate emmetropisation process really is: it may be simpler than expected. In addition to emmetropisation, cephalopods possess accommodation, which also requires a defocus detector. As the cephalopod retina contains only photoreceptors, presumably both short-term defocus requiring accommodation, and longer-term defocus requiring a change in eye growth, is detected and encoded by these cells. How the photoreceptors differentiate the nature and the direction of defocus is not known, although the light sensitive outer segment of the photoreceptor cell takes up a considerable proportion of the retinal thickness. Once the defocus is detected, either the photoreceptor cells themselves release factors that regulate eye growth or change accommodation, or else the signal is encoded in the photoreceptor outputs (as action potentials) and processed externally to the eye.

The comparative difficulty of keeping squid alive in a laboratory environment may hinder widespread adoption of the squid model of emmetropisation. However, the guidelines presented in Appendix 1 can assist with the establishment of new aquaria, and no doubt, the methodology for keeping squid alive in captivity will become more refined over time.

The cephalopod eye presents an anatomically simple and unique model for refractive development, and this study suggests that with further research, cephalopods can contribute to our understanding of emmetropisation in camera-type eyes.
Chapter 12. Appendix: Laboratory husbandry of cephalopods

12.1. Introduction

Before undertaking this thesis, The University of Auckland had no existing facilities for, nor experience with, housing cephalopods. Therefore two new closed system aquaria to concurrently house up to three adult octopus, and raise and maintain two simultaneous populations of squid were established. Most of the design of the octopus aquaria and decisions regarding water parameters were informed by anecdotal advice and discussion with cephalopod enthusiasts on dedicated websites (www.tonmo.com in particular was a very useful source of information). The design of the squid aquaria was guided by the generous advice of teuthologist Dr Steve O’Shea. While not a rigorous scientific description, this chapter should provide sufficient information for others to maintain octopus and squid.

12.2. Octopus aquarium

Maintaining wild-caught adult octopus in a laboratory is relatively common (Leporati, Semmens et al. 2008; Estefanell, Socorro et al. 2010). Successfully hatching octopus eggs in an artificial environment remains challenging, the main obstacle being the ability to able to provide a suitable diet immediately post-hatching (Solorzano, Viana et al. 2009; Hormiga, Almansa et al. 2010). Hatching octopus would have been desirable in our case, but no octopus eggs were found during the course of this thesis work and therefore only adult octopuses were held.

12.2.1. Tank design and water flow

Octopuses were kept in a 940 litre closed salt water aquarium, comprising a 400 litre communal sump and 3 purpose built 180 litre tanks (Figure 12.1). Each octopus was housed individually to prevent cannibalism, and also because mortality rates are reportedly lower when compared to group captivity (Estefanell, Roo et al. 2012). The individual tanks were made from 10mm glass with removable lids, and measured 800h x 550d x 400w millimetres – which was the maximum size that the available space permitted. The tanks were assembled by a local glass worker, and
bound and sealed with aquarium-grade silicone gel, which was allowed to cure for a minimum of 72 hours before filling the tanks with water. Each individual tank had a ten centimetre base of sand collected from the inter-tidal zones of Auckland beaches. Appropriately sized terracotta pots were placed in each tank to provide a habitat and a safe hiding area for each octopus. The layout of the tank was initially designed to assist with the experiments, but after obtaining the octopus, we found that they rearranged the tank contents to their liking, including moving the pots and landscaping the sand. As octopuses are notorious for escaping their tanks\(^1\), each individual tank had a heavy glass lid, which was weighed down with at least one brick to discourage escape. The approximately 4cm gap between the water level and the top of the tank also seemed to act as a deterrent to escape. While octopuses were observed exploring the top of the tank, no attempted escapes were seen.

Figure 12.1 - The octopus aquaria consisted of three individual tanks which housed the octopuses, and a common sump which sat underneath. The sump contained sand beds, aerators, terracotta pots, all the pumps, and a protein skimmer. The eye chart in the left hand tank was used to assess water clarity.

\(^1\) One such example in Dunedin, New Zealand is the recidivist ‘Sid’, who not only escaped multiple times from his tank (including a 5-day holiday living in the drain), but also managed to flood the entire aquarium by removing a valve from his tank. He was eventually released, likely to the relief of the staff.

12.2.1.1. Water inflow

Water was pumped into each tank at approximately 3000 litres per hour through a nozzle in the side of the tank above the water level. A high flow rate was required in case the octopus 'inked' inside the tank, as it would need to be rapidly cleared to prevent suffocation. The inlet nozzle created multiple high speed streams of water which disrupted the water surface, and aerated the full depth of the tank. The high flow rate and position above the water line discouraged disassembly by the occupant and prevented an inadvertent siphon should the pump malfunction.

12.2.1.2. Water outflow

Water outflow was through a full width, 80mm high slit at the back of the tank near the top, which allowed water to flow into an 18 litre overflow box. This arrangement maximised retained water volume in the individual tanks in a situation of water inflow failure, such as with a power failure. A Perspex sheet with multiple rows of 3mm diameter holes was placed between the main and overflow tank, which prevented larger items, including octopuses, from returning to the sump. The full-width slit drain made it unlikely that the octopus could block the entire outflow. Each overflow box had two gravity returns to the sump; each return tube was capable of maintaining sufficient outflow if the other became blocked or air-locked. The water from each individual tank returned to a common sump.

12.2.1.3. Flood Protection

Due to the high volumes of water being moved between tanks, measures were put in place to prevent overflow. The major concern was that a curious octopus could impede the outflow of water through the overflow filter by stretching across it, or that the filter may clog overnight with food scraps or other waste material. This would result in an individual tank overflowing, water loss being limited only by the volume of the sump tank (approximately 400 litres). An empty sump tank would also have had potentially devastating effects for the other tanks in the system.

Each individual tank was fitted with an infrared diode on one side, and receiver on the other, above the highest expected water level. When the water level rose, it absorbed the infrared light, and broke the circuit. This tripped a relay in a custom made remote circuit box, which in
turn cut power to the individual tank pump. If the rise in water level was due to a temporary obstruction, such as the octopus covering the filter, the water would again flow out of the main tank into the overflow once the obstruction was cleared, which permitted the inflow pump to turn back on. A variable rheostat was added to each receiver to fine tune the sensitivity and to prevent it turning off if water merely splashed across its path.

12.2.2. Lighting

The lighting cycle in the aquarium was maintained in a 12:12 light:dark cycle, with a thirty minute ramp-up and ramp-down simulating sunrise and sunset. Light at the top of each individual tank was between 190-210 lux, provided by overhead fluorescent tubes (Starcoat T5/f28W, General Electric, USA). Additional task lighting was provided with compact fluorescent tube desk lamps shone through a diffuser, when required.

12.2.3. Water manufacture and salinification

Laboratory tap water came from multiple sources around the Auckland region. The water is quality tested and treated as necessary at each source, but depending on the balance of supply and demand, the original source of the water arriving at a laboratory tap could not be guaranteed (personal correspondence). Because of this lack of control, the use of chemicals to neutralise any unwanted elements in the tap water would have been variable and time consuming. Instead, tap water was purified through a four-stage reverse osmosis and de-ionization (RODI) unit (PSI-019B-DI, PSI Water Filters, Australia, with 6800 booster pump, Aquatex International Inc, USA). This system forced pressurised water through a network of membranes against its osmotic gradient (leaving the solute behind), then passed it through an ion exchange resin, which stripped ions from the water. Absolute water purity was confirmed by having a total dissolved solids (TDS, a measure of total molecular and ionic chemicals in water) of less than one part-per-million (ppm) of impurity (measured with TDS-4, HM Digital, Los Angeles, USA). Water purity was periodically checked, as a saturated de-ionising gel in the RODI unit would have allowed increasing amounts of ions through. Stringent quality levels were placed on the water in the aquaria, and checked regularly (Table 12.1).
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Acceptable range</th>
<th>Corrective action</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.8-8.4</td>
<td>Add Calcium Buffer</td>
</tr>
<tr>
<td>Temperature</td>
<td>18-25°C</td>
<td>Adjust room air conditioner</td>
</tr>
<tr>
<td>Ammonia</td>
<td>&lt;0.50ppm</td>
<td>Remove food scraps/waste products. Decrease bioload if necessary</td>
</tr>
<tr>
<td>Nitrite</td>
<td>&lt;0.50ppm</td>
<td>Directly related to ammonia, action as above</td>
</tr>
<tr>
<td>Nitrate</td>
<td>&lt;80ppm</td>
<td>Increase fresh water replacement</td>
</tr>
<tr>
<td>Salinity</td>
<td>SG 1.022-1.029</td>
<td>Top up tank water levels with RODI water</td>
</tr>
<tr>
<td>Water volume</td>
<td>Pumps submerged, skimmer exit above water line</td>
<td>Adjust as necessary by adding RODI or bailing water from sump</td>
</tr>
<tr>
<td>Fittings</td>
<td>No water leaks</td>
<td>Tighten or replace parts as necessary</td>
</tr>
</tbody>
</table>

Table 12.1 - Summary of the acceptable aquaria parameters that were checked for both the octopus and squid aquariums twice per week. Further details are provided in the body text.

Commercial aquarium sea salt (a mix of Marine Salt, Red Sea, Israel and Sea Salt, Aqua One, Hampshire, UK) was added as required to achieve a salinity of 1.022 (specific gravity (SG), or 29.2 parts per thousand), measured with a temperature calibrated refractometer (RHS-10ATC, Huake Instrument Company, Shenzhen, China). The salinity of 1.022 represents the lower limit of the desired salinity range, and was chosen because the salinity would be expected to increase with time as water evaporated from the sump. This continuous evaporation also reduced the likelihood of accidental over-dilution of the sump water during routine topping up with RODI water.

12.2.3.1. Tank cycling

A major disadvantage of a closed water system is the need to establish a microenvironment mimicking the biological functions of the ocean, especially with regards to the nitrogen cycle (Yuen, Yamazaki et al. 2009) as animal waste causes accumulation of ammonia in the water. In a closed system, ammonia concentration will slowly increase to toxic levels (Camargo and Alonso 2006). Ideally, ammonia is broken down through oxidation to progressively less toxic compounds by nitrifying bacteria (Knowles, Downing et al. 1965; Young, Thompson et al. 1979). To facilitate this process and before any octopuses were housed, 80 litres of sand was collected.
from various Auckland beaches and added to the sump to serve as a habitat for nitrifying bacteria. The sand was kept in two containers to prevent disturbance from the water flow, and drawing into the pumps. The growth of the required bacteria was encouraged by addition of decaying fish heads to the sump. This initially resulted in very cloudy water, and a rather unpleasant atmosphere in the room owing to the extremely high levels of ammonia. After several weeks, the ammonia levels started to decrease, and nitrite levels, the next stage of the nitrification process, began to rise (Figure 12.2). Eventually nitrate levels increased, and both ammonia and nitrite concentrations approached zero.

![Graph showing the initial 'cycling' of the octopus tank setup, with parts per million of ammonia, nitrite and nitrate on the left, and pH on the right axis.](image)

Figure 12.2 - Graph showing the initial 'cycling' of the octopus tank setup, with parts per million of ammonia, nitrite and nitrate on the left, and pH on the right axis. On 5th May, a large fish head was added to the system and allowed to decay. Ammonia quickly rose to toxic levels (>0.5PPM), and the water began to acidify. After 20 days, both nitrite and nitrate levels increased, indicating the establishment of the next stage of the cycle. Nitrite is slightly less toxic than ammonia, but gets quickly utilised by the nitrate forming bacteria. Nitrate is much less toxic, and safe levels can be as high as 80PPM. The decrease in nitrate levels from the 10th June indicated establishment of anaerobic nitrifying bacteria in the sand beds. Once nitrate levels decreased, pH returned to baseline levels, although this may also be confounded by the addition of oyster shell, with the calcium carbonate acting as a pH buffer. By 15th June 2009 all parameters were within our limits for safe occupancy by an invertebrate.

The tank is said to have cycled when ammonia and nitrite return to zero, and indicates the water is ready for marine life to be added. During the establishment of bacteria, water quality was measured daily using a commercial salt water testing kit (API Saltwater Master Liquid Test Kit,
Mars Fishcare Inc, Pennsylvania, USA, which allowed measurement of ammonia (NH$_3$), nitrite (NO$_2^-$), nitrate (NO$_3^-$) and pH.

Stable nitrate levels are typically considered the end stage of the aquarium nitrogen cycle, as the next stage of the nitrogen cycle, denitrification of nitrate to nitrogen gas, requires heterotrophic bacteria such as *Pseudomonas fluorescens* (Casasús, Hamilton et al. 2005) or *Paracoccus denitrificans* (Davis, Doudoroff et al. 1969). When these bacteria metabolise under anaerobic conditions, they are able to use nitrate, instead of oxygen during respiration. However, anaerobic bacteria are hard to cultivate in a shallow, closed-water system, particularly as the octopus required highly oxygenated water. Fortunately, over time the nitrate levels naturally decreased, which was attributed to the 10 centimetre bed of sand, shells, and rocks in the individual tanks, and the sand bed in the sump, which may have allowed a shallow anaerobic zone (Yuen, Yamazaki et al. 2009). The presence of bacteria was not always sufficient, and occasionally water replacement with artificial salt water was required to keep the nitrate level within an acceptable range. Once the tank parameters had stabilised, water testing was decreased to twice weekly, but returned to daily after the addition of a large amount of biomass (such as a new octopus), until it had again stabilised.

Ammonia is not the only waste product that accumulates in a marine aquarium; there are also a range of larger inorganic molecules from food, including amino acids and proteins (Huang, Dong et al. 2011). Therefore a protein skimmer was added to the sump (EV-400, Aqua C, USA). Protein skimmers mimic the effect of sea foam on a surf beach by utilising a process called foam fractionation. This process can very quickly remove large quantities of nitrogenous and ionic waste (Hussenot, Lefebvre et al. 1998). Our protein skimmer used a counter-current spray induction design, chosen as it was adjustable to our likely total water volume, and the simplicity of the design appeared compatible with long term, maintenance-free use. It used pressurised water pumped through a specially designed nozzle suspended slightly above the sump water level to force water and trapped air (from the gap between the nozzle and the water) down into the skimmer unit. Air bubbles attract hydrophobic and amphipathic molecules such as proteins and lipids, which form around their surface, allowing the bubble to persist above the water surface (Valdes-Krieg, King et al. 1975). With enough protein, the bubbles rise into a central chamber (Stevenson, Li et al. 2008) and form a foam base, which eventually rises through pneumatic pressure at the base (Stevenson 2007) and spills over into a collection cup near the
top of the chamber, liquefying back into a concentrated solution (Neethling, Lee et al. 2003). The cup prevents the ‘skimmed’ molecules from re-entering the water, and was periodically emptied to remove the waste.

12.2.4. Oxygen

Oxygenation of the water is an important factor as octopuses have a high oxidative demand (Farias, Uriarte et al. 2009). However, it was not measured, but assumed to be constantly high with the presence of visible air bubbles throughout each individual tank as a result of the high pressure water inflow, the waterfall outflow into the sump, and the heavy aeration provided by the air-injection protein skimmer.

12.2.5. Temperature

Water temperature was maintained between 20-25°C Celsius by controlling the room air temperature with an air conditioner. Water temperature was measured twice per week and was a nearly constant 23°C. As octopuses are cold blooded, their growth rate is largely dependent on their food supply (Opresko and Thomas 1975; Forsythe and Hanlon 1980; Cortez, González et al. 1999) and water temperature (Forsythe and Hanlon 1988; Miliou, Fintikaki et al. 2005). This temperature (23°C) is within the range typically found in other laboratory aquaria. However, this is slightly warmer than the sea-water where our octopuses were collected (17.5°C to 21.0°C, periodically self-measured throughout the year) and lacked a natural seasonal variation. As such, an acclimatisation period was employed when introducing an octopus into the aquarium. Aquarium water replaced the water in the bucket containing the octopus at approximately 15% every 5 minutes, for at least 25 minutes.

12.2.6. pH

The pH of the water in the octopus aquarium was measured twice weekly using a digital pH meter (Q1416, DSE, Australia), which was calibrated in a pH = 7.0 buffered solution once per month. Calibration error was at most 0.1pH units towards alkaline, which would tend to underestimate the acidity. pH was maintained at a mildly alkaline level of between 8.0-8.4, identical to the range measured at the capture location. High concentrations of nitrates tend to make the water acidic, so the water needed to be buffered to prevent pH swings during feeding.
and excretion (Grguric, Sondey et al. 2000). The water in our aquarium was heavily buffered with commercial blends of carbonate and bicarbonate salts (KH+, Red Sea, Israel, and Alkaline Buffer, Seachem, USA). Oyster shells were also added to the sump tank to act as a natural source of carbonate.

12.3. Squid Aquarium

As discussed in Chapter 3, raising squid from eggs is still considered an emerging field, and has only been successfully achieved in a limited number of species. I was fortunate to have advice and assistance from Dr Steve O’Shea, a marine biologist with a personal interest in cephalopod husbandry. He loaned the initial equipment, provided advice and troubleshooting, and demonstrated areas around Auckland for catching suitable food for the growing squid. Additionally, he supplied two egg masses, one of which successfully hatched and was used for Experiment 1 (Chapter 5).

12.3.1. Tank design

The squid aquarium system consisted of two 500 litre vertical cylindrical tanks, each measuring 0.8 meter in diameter, and one meter in height. A corner-less design was required, as recommend by Yang et al (Yang, Hanlon et al. 1989) and discovered through personal experience: in a square tank the squid invariably meet a corner and seem to get ‘stuck’. The cylindrical tanks each had a 30 millimetre diameter gravity outflow located five centimetres from the top of the tank, which drained into a common 180 litre sump tank. Overflow boxes were not required, as unlike octopus, squid do not attempt to escape or disassemble tank fittings. However, filters were placed over the tank outflows to prevent small wayward squid flowing out with the water. The size of the filter mesh was increased as the squid grew, to minimise the risk of blockage.

12.3.2. Water flow

Water was forcibly injected into each tank through a pressure spray-bar with an adjustable angle, located above the water level. This allowed control of surface disruption and water oxygenation, and permitted the generation of a circular current within each tank. A riser, a long
cylindrical tube with a bubbler at the bottom, was situated adjacent to the outflow in each tank, running the full height of the tank in order to establish a vertical water current to draw excess proteins to the surface, which could then be drained to the sump for extraction with a skimmer. Water turnover at maximum flow was 600 litres per hour, although it was usually significantly less than this in order to prevent damaging the paralarval squid. Additional pumps in the sump kept the water turbulent, preventing surface stagnation.

12.3.3. Lighting

The lighting cycle in the aquarium was maintained in a 12:12 light:dark cycle, with 30 minutes ramp-up and ramp-down (simulating sunrise/sunset), for the entire pre-hatching and post-hatch period. Light levels are known to exert an effect on the incubation period of marine eggs, such as fish, but this does not appear to be true for squid eggs, at least between 2.5 and 600 lux (Sen 2004). Hatchling survival rates may be inversely proportional to lighting intensity (Sen 2004) (squid prefer dimmer environments), although Sen’s study was poorly controlled for other variables, such as temperature, which fluctuated widely and included colder temperatures known to negatively affect embryogenesis in squid (Sen 2005). Bearing this in mind, direct illumination was not used, other than for feeding and cleaning the tanks.

12.3.4. Water acquisition

No artificial sea water was used in the squid aquarium, in order to provide as natural environment as possible and to save time in setting up the system. Salt water in the squid aquarium was obtained from the Waitamata Harbour, off Auckland’s East Coast. Natural seawater was chosen to ensure appropriate local micronutrients in the water, which may be required for proper embryo development. This also had the effect of speeding the cycling time of our new setup by seeding bacteria. Water was exchanged at approximately 30 litres per week, or as required, with fresh sea water.

12.3.5. Water temperature

The sump water ran through an external refrigerator unit (Chiller: HC-300A, Hailea, China with pump: 3400, Eheim, Germany), which maintained water between 18° to 19° C, which approximates summer water temperatures off the New Zealand coast (Uddstrom and Oien 1999).
Hatching rate (number of hatching eggs / number of incubated eggs) is reportedly high between 12° to 22° C, but hatching success (number of viable paralarvae / number of incubated eggs) requires a narrower range of 16° to 22° C to achieve 85% success rate (Sen 2005).

12.3.6. Water filtration

As the squid aquarium was a closed system, care had to be taken with nitrogenic waste products, particularly when housing a large number of animals. Water quality was maintained at the same level as the octopus aquarium (Table 12.1). As the tank had no sand bed, there was significantly less potential biofiltration available in the system compared to the octopus setup. To compensate for this, two biofilters (Eheim 2217 Canister Filter, Eheim Aquatics Group, Germany) were added to the system. These allowed water to pass through a substrate chamber which housed various sized filters and highly porous beads, which provided a large potential surface area for nitrifying bacteria to grow on (Eheim GmbH & Co. 2012).

Excess waste products and debris were netted and vacuumed from each tank at least once a day, after the squid had finished feeding. Nitrate testing was performed twice weekly once the eggs had hatched.

12.3.7. Salinity

As natural sea water was used, salinity was relatively constant throughout the experimental period, and only varied with the evaporation of water from the common sump tank (range: specific gravity (SG) at 19° C: 1.027 – 1.028 (36.5 – 38.0 parts per thousand)). Unlike inter-tidal octopus, squid are unlikely to experience dramatic changes in salinity while at sea and may be poorly equipped to survive sudden changes (Steer, Moltschaniwskyj et al. 2002; Pecl and Moltschaniwskyj 2006). Evaporation of water increased salinity, and was controlled through the addition of RODI water to the sump. The effect of various salinities on squid hatching rates has been investigated (Sen 2005), with very high (min 92%) hatching rates from SG 1.023 – 1.030, and the highest hatching success rates (min 92.7%) between SG 1.025 – 1.028. Salinity of water in the squid tanks was tightly controlled between these values throughout the experiment, measured every second day with a temperature calibrated refractometer.
12.3.8. pH

pH was measured using a digital pH meter. As natural sea water was used, it was naturally pH buffered and slightly alkaline. Yang et al (Yang, Hanlon et al. 1989) recommend pH remain above 7.8 and this was set as the lowest acceptable value. Squid have a deep sea habitat, where sudden shifts in acidity are unlikely to occur, and it was unknown how well squid would adapt to any sudden changes. As such, sufficient alkaline buffer was kept in stock, and pH was tested twice per week. Fortunately, it remained at 8.2 throughout the experimental period. An acidic shift could have been indicative of an insufficient buffer, or excess nitorgenic waste. Protocol dictated that if the pH dropped to 8.0, tank cleaning frequency increased, and the water replacement schedule was also increased.

12.4. Pre-hatching incubation of squid eggs

12.4.1.1. Capture

Squid egg masses were collected from reefs, or opportunistically collected from the beach around the greater Auckland region. Egg-masses were immediately transported to our aquarium in 20 litre buckets of sea water, with an aerator in the water, or with frequent stops for water replacement as required. The water in the bucket carrying the egg masses was gradually mixed with existing aquarium water over a period of 30 minutes, to allow the eggs to gradually acclimatise.

The eggs of *Sepioteuthis australis* can be laid down year-round, although laying generally peaks in late spring and early summer (Moltschaniwskyj and Pecl 2003). The eggs are laid down in batches, the female appears to save several eggs to be laid down at once (Pecl 2001). Each embryo is contained with a pod, with each pod containing between 3-9 eggs. Each pod is individually attached at one end to a thick fibrous core. The total egg mass can contain a large number of pods; Steer reported a single egg mass containing 1241 egg strands (Steer and Moltschaniwskyj 2007). A single egg mass may contain eggs deposited by several different females (Jantzen and Havenhand 2003). Once laid, they are attached to a stalk of kelp or other fixed underwater objects, and they move around with local water eddies. They can commonly be seen attached to the stem of costal *Ecklonia radiata* (Agardh, JG 1848) in New Zealand (Figure
12.3) or *Amphibolis antarctica* (Sonder & Ascherson ex Ascherson 1868) off the coast of Australia (Moltschaniwskyj and Pecl 2003; Moltschaniwskyj and Steer 2004). The egg masses we collected included from 7 to approximately 200 strands. Similar to other cephalopods, shortly after laying eggs the mated animals cease eating and die within a week, prior to their offspring hatching (Triantafillos 2001).

Size of hatchlings appears to decrease later in the season, peaking around November and decreasing through to February. Steer (Steer, Pecl et al. 2003) suggests increasing water temperatures as a possible cause, although there is also evidence (Steer, Moltschaniwskyj et al. 2003) that cooler waters can produce larger size. As their natural environment is complex and varied, it is possible to expect large seasonal variation of hatching size, dependent on numerous factors, such as rainfall, chemical composition of the water, total sunshine hours, and variations in water temperature.
12.4.1.2. Egg mass incubation

The incubation period in squid shows substantial variability between species, and is somewhat inversely related to water temperature (see the Introduction of (Sen 2004) for a concise summary). At 18°C, the incubation time for *Loligo vulgaris* is 23-29 days (Sen 2005).

Most of our egg masses were obtained at an early embryological stage, and were incubated in the aquarium for periods ranging from 3 to 20 days before hatching. The masses were attached to light-weight aquarium tubing, suspended in the tank, and placed in mild water current. Initial incubations had poor survivability, presumably due to poor gas exchange in the large egg masses. Subsequent masses were divided into smaller masses (approximately 50 pods), and placed near a submerged airstone bubbler to ensure high oxygenation in the core of each mass. The water inflow nozzle was adjusted (45° to the surface) to provide maximum aeration and a strongly rotating water flow, which caused the egg mass to move in the current. With early trials, the eggs were also handled and inspected daily, including massaging, to prevent stagnant water remaining trapped in the egg mass core, and to rinse off surface debris which tended to gather on the proximal tips of the capsules. Early attempts at keeping the egg mass alive until all capsules had hatched were only moderately successful: part of the reason was believed to be the cessation of this massage once hatching began. To rectify this, and to better simulate the conditions in which the eggs would normally hatch, a wave simulator was built. The egg masses were suspended in middle of the tank by aquarium tubing. One end of the tubing was fixed to the edge of the tank, with the other end fixed to a rod extension attached to the body of an oscillating fan. The oscillations of the fan caused the egg mass to gently rise and fall within the tank. The fan was set to run at low speed for approximately one hour every 75 minutes, which ensured that water moved through the egg mass, and also changed the depth and therefore pressure, and was intended to simulate the regular waves or ocean surge that would pass through the eggs in the wild.

*Sepioteuthis australis* hatch post-larval from encapsulated pods as miniature adults. Unlike the octopus, squid lack a true planktonic larval stage, and hatch as independent individuals which must quickly learn to actively hunt prey in order to survive (Yang, Hanlon et al. 1983).
12.4.1.3. Embryo Survivability

Embryos at the proximal end of the pod (i.e. towards the ‘core’) tend to be less developed, and have lower survival rates than more distal embryos (Steer and Moltschaniwskyj 2007). Additionally, embryo survivability can be improved by keeping the size of egg masses less than 100 pods (Steer and Moltschaniwskyj 2007). Proximal embryos on the innermost egg pods of the egg mass have mortality rates as high as 95%, compared to 4% for distal peripheral pods (Steer and Moltschaniwskyj 2007). Fewer strands presumably allows better exchange of nutrients in and out of the proximal capsules, although mechanical compression of the embryos may also play a part.

Biofouling was commonly seen in our laboratory-cultured eggs, even developing when the eggs were clean when brought into the tanks. This was allowed to continue at a moderate level, and taken to mean the egg masses were healthy, as there is reportedly a negative correlation between increased biofouling and squid mortality, up to a point (Gowland, Moltschaniwskyj et al. 2002; Steer and Moltschaniwskyj 2007). Occasionally biofoul material was inside non-hatchling embryos, although it was also often seen inside late stage embryos that were obviously alive, therefore it was unclear whether or not this played a role in the embryo mortality. Each embryo is protected from the surrounding sea by two layers, the egg ‘shell’, and the outer collagenous layer of the pod. Interestingly, these protective layers fail to develop normally in Dosidicus gigas (Humboldt Squid) that have been artificially fertilised (Staaf, Camarillo-Coop et al. 2008), and it is not clear if the improper formation of these protective layers allows the biofoul material to get inside the egg capsule.

Season of birth may also play a role in embryo survivability, although this may be due to changes in rainfall and surface salinity, rather than temperature differences (Steer, Moltschaniwskyj et al. 2002).

12.4.2. Embryo development

S. australis embryological development can be divided into 30 stages, with hatching occurring at stage 30. Each stage is based on the appearance of features within the embryo, and has been previously well described (Steer, Moltschaniwskyj et al. 2003). We found squid hatching at less
than stage 28 were unable to survive, as the size of the remaining yolk was too large for them to swim. Removal of the yolk did not appear to improve survivability.

12.4.2.1. Hatching

Once hatching was imminent, indicated by an extremely nodular and transparent appearance of each pod, the water inlet nozzle was adjusted to vertically inject water (10° from the normal) to allow oxygen exchange and disruption of the surface, without creating a circular current or submerged bubbles which may have posed a threat to the juvenile squid. Total hatching periods can last from six to eight days (Sen 2005), however, we found the egg mass began to deteriorate (rot) from the time when the earliest hatchlings emerged. As our egg mass was suspended in the same water system as we intended to grow the squid and we wanted to expose the hatchlings only to pristine water quality, the egg mass was removed 48 hours after the earliest sign of deterioration was apparent, by which time the hatching rate of the remaining squid had greatly decreased. The agitation associated with removing the egg mass caused a spike in hatchling numbers, but it is likely that a higher proportion of squid could have hatched given more time.

We found that manual manipulation of the egg mass, particularly near hatching, also caused premature hatching (stage 28 or earlier; hatchlings with visible egg yolks still attached, which were unable to swim or hunt normally quickly died). Consequently, the wave simulator remained on throughout the hatching period, and the continual wave motion seemed to provide mechanical assistance for the hatchlings to escape the egg capsule, without stimulating premature hatching.

12.5. Feeding

Providing adequate and appropriate food is one of the biggest challenges to raising squid in a lab environment, and as such it demands a reasonable space in this appendix. It must be noted that the location and techniques for catching food was demonstrated to us by Dr Steve O’Shea, who was Director of the Earth and Oceanic Sciences Research Institute at the Auckland University of Technology. The capturing of food was conducted by myself, and my supervisor Dr John Phillips, who often ventured into the swamps alone, in the name of science.
Squid are unusual amongst the mollusc phylum, in that they possess a prenatal yolk, which provides energy for the paralarvae to both find food and acquire the necessary skills to begin hunting (Moltschaniwskyj, Hall et al. 2007; Martins, Roberts et al. 2010). At 18° C, (similar to the temperature of our experimental setup) the yolk sac was expected to provide sufficient reserves to enable survival for up to 5-6 days without successfully capturing prey (Martins, Roberts et al. 2010).

Immediately post hatching, hatchlings can theoretically feed on live prey, although observation showed that they took at least one day to obtain sufficient co-ordination and technique to catch prey. Those born slightly prematurely may also have residual yolk sac in their oesophagus, likely a physical hindrance to catching more food. Squid are selective in their food preferences, requiring something alive and of approximately the same size as themselves, and rejecting anything significantly larger, smaller, or dead. An additional concern was the inability of the juveniles to defend themselves, making prey selection within the confines of a closed tank system vital to their survival. For the first 20 days post-hatching, the juvenile squid were provided very small mysid (Tenagomysis novaezelandiae, Table 12.2) to hunt. These were caught every couple of days from intertidal mudflats in the central Auckland area (Hobson Bay, -36°.86’S, 174°.81’E) using hand nets at low tide. Stock populations were kept alive in the lab with a tank aerator. The squid were fed twice per day, until they stopped hunting additional food. More information on the biology of mysid species is given below. By 20 days post hatching, the squid mantle length had grown to approximately 10 millimeters, so the food was switched to a larger species of mysid, Tenagomysis chiltoni, and small palaemonid shrimp, Paratya curvirostris, were occasionally introduced. These were caught with hand nets from tidal streams around Cornwallis, on Auckland’s Northwest coastline (-37°.00’S, 174°.59’E). From day 60 onwards, squid were also fed small live fish, including Yellow Eyed Mullet (Aldrichetta forsteri) and Mosquito fish (Gambusia affinis).
Table 12.2 - Types of food that the developing squid accepted. The days were not fixed, and the food sources were gradually exchanged, providing time for squid to modify their hunting behaviour for the new prey. During the times when new food was being introduced, effort was made to preferentially select smaller prey.

All excess waste was skimmed from the tank once feeding had completed, to minimise nitrogenic waste.

12.6. Prey

The variety of prey that was found to be suitable for the squid were native species; however detailed information is provided so that an equivalent match may be made outside of New Zealand.
12.6.1. Mysida

The largest challenge of raising squid was finding sufficient food suitable for the squid to hunt during the immediate post-hatching phase. The food needed to be of sufficient interest and of an appropriate size, while being passive or ineffective enough to not pose a threat while the squid were learning how to hunt. We used freshwater mysid caught in intermittently closed and open lakes and lagoons (ICOLLs), as these could be caught by hand.

Mysid are small shrimp-like creatures, found in fresh, brackish, and marine waters (Jones, Greenwood et al. 1989; Azeiteiro, Jesus et al. 1999; Roast, Widdows et al. 2000; Drake, Arias et al. 2002; Marcelo Acha, Mianzan et al. 2008). Mysid comprise a substantial component of the zooplankton around the world, and bridge the food chain between primary microbial producers (bacteria, plankton) and secondary feeders, like fish (Hostens and Mees 1999; Roast, Widdows et al. 2000; Roast, Widdows et al. 2004; Carleton and McKinnon 2007). The Mysida order is colloquially called ‘mysid shrimp’, however they are distinct from true shrimp in that they carry their relatively small brood of eggs (Jones, Greenwood et al. 1989) in a marsupium (a pouch), giving them the common name of opossum shrimp. T. novaezelandiae is often used as a test species in survival studies of the effect of chemicals in the marine environment (2010).

The mysid were sourced from fresh to brackish water in ICOLLs in the Auckland region. A small mesh (1-1.5mm square mesh size) aquarium hand net (20cm wide) was trawled along the bottom of the muddy or sandy stream beds at low tide. The net was ‘jiggled’ along the bottom in order to stir up mysid which had buried themselves. Catches had highly variable success rates – with some hunts being complete in 30 minutes, while others required 4 or more hours, spread across two daily low tides. Care was taken to avoid by-catch (small flat fish, or larger shrimp species), although this was not always possible due to the murkiness of the water and difficulty in differentiating a juvenile shrimp from an adult mysid. Population depletion was of concern, so excess catching was avoided: the maximum catch of mysid at any time was restricted to two densely populated 20 litre buckets (estimated to be 2000 mysid in total).
Figure 12.4 – Tidal mudflats (ICOLL) at Hobson Bay, Auckland, during low tide. The high tide mark is along the tree line visible in the distance. The bed of the river was a mix of empty shells, mud, and sand. *Tenagomysis novazealandiae* were caught along the banks of this stream.

The squid were fed two types of mysid shrimp, *Tenagomysis novazealandiae* and *Tenagomysis chiltoni*, the only two species of mysid found in freshwater environments in New Zealand (Chapman 1976).

The mysid were caught and transported to the lab in 20 litre opaque buckets with lids. Once back in the lab, they were stored in water collected from the area, primarily the Hobson Bay inter tidal mudflats for *T. novazealandiae*, and the feeder streams of the Kakamatua Inlet for *T. chiltoni*. Testing of the water in the laboratory showed wide variation of salinity and temperature, based on proximity to high tide, tide height and recent rainfall, and observation showed great variation in the turbidity of the water. In the lab, the water temperature settled to room temperature (controlled at 24°C), and water oxygenation was maintained through the use of a submerged aerator. A bed of debris accumulated over time, composed of debris caught at the catchment area, dead shrimp, waste products, and excess food; this was cleaned out infrequently (approximately every two weeks). Salinity was not adjusted, although it did vary with the delivery of new feeding stock and water. The mysid were fed tropical fish flakes (Brine
Shrimp Flakes + Freeze Dried Brine Shrimp Nutrafin MAX, Hagen, Canada), and the water was not filtered, instead being partially replaced when new mysid were collected from the ICOLLs. There was no significant observed mortality over the range of conditions they were kept in, even with the sometimes sudden changes in water parameters. Food ingestion/egestion may have varied with temperature and salinity (Roast, Widdows et al. 2000), but this was not measured nor monitored as the maximum captivity of a single mysid was relatively short (food gathering was conducted daily to once every 3 days, depending on catch rate and weather).

The first mysid fed to the squid, T. novaezelandiae (Thomson, 1900), is a relatively non-aggressive mysid ranging in size from 1.75 millimetres as juveniles, up to 9.91 millimetres during pregnancy (Jones, Greenwood et al. 1989). Our catches tended to be of 4-8 mm mysid, as our net mesh size possibly let the more juvenile mysid pass through lengthwise. Two New Zealand studies have investigated the life cycle of T. novaezelandiae, but were unable to find evidence for a seasonal breeding or maturation cycle (Jones, Greenwood et al. 1989; Lill, Lal et al. 2010). Interestingly, both studies showed a peak in total catchment numbers in March, the end of the New Zealand summer, which may indicate summer gestation.

After 15 days of feeding on T. novaezelandiae, the squid were gradually transitioned onto the larger T. chiltoni mysid. These mysid are slightly larger, ranging from 2.0mm as juveniles to 18.6mm as adults, and could be more aggressive in their behaviour towards the smaller squid (Jones, Greenwood et al. 1989). They are more sensitive to gradients in salinity (Jones, Greenwood et al. 1989), and were found in fresher water (approximate SG 1.008). The transition from one prey to the other took place over 5 days, and the T. chiltoni were initially screened for size, feeding the squid the smaller of the species during the transition period. T. chiltoni occupy similar ICOLLs to T. novaezelandiae, although they appeared to prefer less salty water, as previously reported (Lill, Lal et al. 2010).

12.6.2. Shrimp

After the squid grew significantly larger than the largest mysid, they were gradually introduced to a diet of shrimp, including Paratya curvirostris and Palaemon affinis. Shrimp, like mysid, are found in almost all aquatic environments, including fresh water, ICOLLs, and deep sea environments.
*P. affinis* is an abundant shrimp which occupies the intertidal zones. While initially thought to be circumpolar, it is now considered endemic to New Zealand (Day 2001). They can occupy a wide range of water variables, including salinity ranging from almost fresh to marine, and even stronger given time to adjust (Kirkpatrick and Jones 1985). Once captured, they were stored in an unoccupied octopus tank and fed on tropical fish flakes (Brine Shrimp Flakes + Freeze Dried Brine Shrimp Nutrafin MAX, Hagen, Canada).

A second type of shrimp, *Paratya curvirostris* (Heller, 1862), is New Zealand’s only freshwater decapod shrimp. Most of the research conducted on *P. curvirostris* comes from Carpenter, who conducted his Master’s thesis on its biology. *Paratya curvirostris* appears almost translucent, and demonstrates sequential hermaphroditism (protandry – male to female) and the size distribution is unequal between the sexes. The larger females are able to carry more eggs (between 900-4000), which are carried until maturity at 28 days. There is weak evidence that post hatching, larvae drift downstream to brackish water, which is consistent with most other freshwater shrimp, before returning to fresher water to complete the life cycle (Carpenter 1983).

*P. curvirostris* was collected from rivers and upstream of ICOLLS, where the salinity ranged from SG 1.05-1.01 (6.6-13.3 ppt). They were so abundant that sufficient quantity could be collected by dragging two hand nets (2mm mesh, 20cm diameter) near the surface of the water for approximately 15 meters. Catches were particularly bountiful under ledges, where shrimp were seen leaping from the surface of the water in the bow wave of the net. The nets were emptied into 20 L opaque buckets filled with water from the location, and immediately transferred back to the lab.

In the lab, these shrimp were kept in the original buckets that they were captured in as there were no freshwater facilities available. An air pump was added to each bucket, as despite their tolerance to low oxygen, stocking density was high. The shrimp were fed enough tropical fish flakes so that some remained un-caught and collected on the bottom of the bucket. Surprisingly, no ‘shock’ was noted when they were transferred to the squid tanks (which were both saltier and 5°C cooler), which may be attributable to their lifecycle described above. The shrimp were immediately evasive of the squid, preferring to settle near the bottom of the tank.


