Ontogenetic changes in retinal ganglion cell distribution and spatial resolving power in the brown banded bamboo shark, *Chiloscyllium punctatum* (Elasmobranchii)

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Running title: Development of retinal sampling in *C. punctatum*

Figures: 7 (5 colour)

Tables: 2

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Key Words: retina, development, elasmobranch, shark, topography, apoptosis, embryogenesis, *Chiloscyllium punctatum*, ganglion cells

Abstract

The development of the visual system in anamniotic vertebrates is a continual process, allowing for ontogenetic changes in retinal topography and spatial resolving power. We examine the number and distribution of retinal ganglion cells in wholemounted retinae throughout the protracted embryonic development (approx. five months) of a chondrichthyan, the brown banded bamboo shark, *Chiloscyllium punctatum* from the beginning of retinal cell differentiation (approximately half way through embryogenesis) to adulthood. We also identify and quantify the number of apoptosed cells within the ganglion cell layer to evaluate the contribution of apoptosis to changes in retinal topography. *C. punctatum* undergoes rapid changes in ganglion cell distribution during embryogenesis, where high levels of apoptosis especially around the retinal periphery, result in relative increases in ganglion cell density in the central retina, which progressively extend nasally and temporally to form a meridional band at hatching. Post-hatching, *C. punctatum* forms and maintains a horizontal streak, showing only minor changes in topography during growth, with basal levels of apoptosis. The total number of retinal ganglion cells reaches 547,881 in adult sharks, but the mean (3,228 cells mm⁻²) and peak (4,983 cells mm⁻²) retinal ganglion cell densities are highest around the time of hatching. Calculated estimates of spatial resolving power, based on ganglion cell spacing (assuming a hexagonal mosaic) and assessment of the focal length from cryosections of the eye, increase from 1.47 cycles degree⁻¹ during embryogenesis to 4.29 cycles degree⁻¹ in adults. The increase in spatial resolving power across the retinal meridian would allow this species to hunt and track faster, more mobile prey as it reaches maturity.

Introduction

Chondrichthyan pups are born with no parental protection or support, instead relying on a suite of developed and functional sensory modalities for survival. These sensory abilities allow the pups to interpret their surroundings, avoid predators and locate and capture prey. However, while a great deal of information exists on chondrichthyan sensory capability post-hatching, very little information currently exists on sensory development during embryogenesis to ensure the functionality of these essential systems at birth.

As with other vertebrates [Hollyfield, 1972, Kahn, 1974, Sharma and Ungar, 1980, Young, 1985, Holt et al., 1988, Harman and Beazley, 1989, LaVail et al., 1991, Hagedorn and Fernald, 1992, Cheon et al., 1998, Olson et al., 1999, Schmitt and Dowling, 1999, Rapaport et al., 2004,], ganglion cells are the first cells to differentiate in the chondrichthyan retina [Fishelson and Baranes, 1999, Harahush et al., 2009]. They are generated at the retinal periphery and are often added non-uniformly, creating topographic variation in density as the retina continues to grow throughout life. Areas of increased cell density subserve increased spatial resolving power within localised regions of the visual field and the distribution or topography of retinal ganglion cells (RGCs) is species-specific and can be highly reflective of an animal's habitat and lifestyle. Traditionally, ganglion cell topography has been considered to be based primarily on differential cell addition, but is also likely due to (differential) cell death [Coleman et al., 1984, Dunlop et al., 1987, Lia et al., 1987, Provis, 1987, Beazley et al., 1989, Bozzano and Catalan, 2002].

Retinal topography is often associated with ecological niche; and predominantly habitat [Hughes, 1977] and inter- and intraspecific interaction (predation, predator avoidance,

reproduction). Two main patterns of RGC topography are recognised in vertebrates: the horizontal streak and the *area centralis*. A horizontal streak is common in animals inhabiting visually open environments and provides individuals with a broad view across a horizontal plane, enabling for the detection of predators, prey and conspecifics with minimal head movement. Alternatively, single or multiple *area centralis* are commonly found in animals occupying complex visual environments, or those which utilise rapid targeted or ambush predation.

Despite the vast number of studies assessing RGC topography in adult vertebrates, knowledge of the embryonic development of RGC distribution is sparse. Many vertebrate models are difficult to obtain as embryos or have rapid gestation. Amniotic vertebrates (i.e. mammals and birds) do not have continuous cell replication and differentiation throughout life; however, teleosts and amphibians possess continuous retinal cell division and differentiation throughout life [reviewed in Fernald, 1989]. In members of these anamniotic vertebrate classes, the retina grows by the continuous addition of proliferative stem cells, situated within the retinal germinal zone [Johns and Fernald, 1981, Fernald, 1989, Hagedorn and Fernald, 1992].

Retinal apoptosis, or programmed cell death, is a naturally occurring physiological process in the development of multicellular organisms [Vecino et al., 2004]. While cell death in the developing retina is common among vertebrates, the timing and magnitude of retinal apoptosis varies between species [Vecino et al., 2004]. It has been suggested that anywhere from 50-90% of RGCs die during development [reviewed in Dreher and Robinson, 1988, Linden and Reese, 2006], typically over two or more phases [Glücksmann, 1940, Harman

and Beazley, 1989, Gaze and Grant, 1992, Biehlmaier et al., 2001, Cole and Ross, 2001, Vecino et al., 2004].

Most studies conclude that cell death plays a role in the formation of non-uniform RGC topography [Sengelaub and Finlay, 1982, Provis, 1987, Wong and Hughes, 1987b]. Cell death occurs predominantly in the retinal periphery, which usually represents the area of lowest cell density, thereby highlighting the contribution of cells to the retinal regions of high density [Sengelaub and Finlay, 1982, Dunlop and Beazley, 1987, Dunlop et al., 1987]. This suggests that cell death in the periphery gives rise to more central high density areas by virtue of the presence of a higher cell gradient. Ganglion cell topography in mammals is relatively uniform in early development and variation in density emerges only after waves of retinal apoptosis [Stone et al., 1982, Cuadros and Ríos, 1988, Harman and Beazley, 1989, Robinson et al., 1989, Cook et al., 1998, Marín-Teva et al., 1999].

In order to examine the ontogenetic processes involved with the development of the visual system in early-diverged vertebrates, we examined the development of RGC distribution in a species of chondrichthyan that has recently been adopted as a model species for developmental studies [Harahush et al., 2007, Harahush et al., 2009]. The brown banded bamboo shark, *Chiloscyllium punctatum* is a relatively small, hardy shark, which is also a common aquarium species. The commonality of juvenile and adult stages of this shark in the wild and access to eggs with known deposition dates through captive breeding programs make this species an ideal model. The species' relatively protracted gestational period of approximately five months (at 21- 24 °C), early evolutionary divergence and continual growth of neural tissue also makes *C. punctatum* an ideal model for investigations of developmental changes in retinal sampling and spatial resolving power. Developmental

ecology of *C. punctatum* is incomplete; however it is known that this species is generally a benthic, nocturnal suction feeder, eating predominantly polychaete worms, prawns and crabs [Unpublished observations; J. Stead, personal communication]. Post-hatching changes in neuronal tissue are often linked to changes in an animals' environment or diet, however, documented information on the ontogenetic natural habitat preference of this shark is sparse [White and Potter, 2004]. Anecdotal information shows that there is overlap in the habitat preference of this species during later life stages, however, very few embryos or pups have been recorded.

In aquatic non-mammalian vertebrates, ontogenetic changes in eye growth, retinal topography and the spacing of RGCs alters the spatial resolving power of the eye, with concomitant improvements in spatial resolving power occurring with increases in lens diameter [Fernald, 1984]. Continuous cell addition, an increase in the posterior nodal distance of the growing eye and an increase in lens size, all allow an animal to maintain, and often increase, their visual ability during growth [Johns and Easter, 1977, Johns and Fernald, 1981, Fernald, 1984, Neave, 1984, Miller et al., 1993, Pankhurst, 1994, Shand et al., 1999]. Ontogenetic increases in acuity provide an animal with the visual potential for improved predator avoidance and prey capture.

This study presents the first systematic examination of the ontogenetic changes in RGC topography during embryogenesis and throughout the post-hatching lifecycle of a chondrichthyan. Specifically, we aim to assess the developmental changes in RGC quantities, densities and patterns and the importance of apoptosis in the formation of topographic patterns of cell distribution from the time of ganglion cell differentiation through to adult

stages. Functional aspects of changes in RGC spacing are also assessed by calculations of the spatial resolving power.

Methods

Animal collection

Animals were obtained from both captive (donated by UnderWater World, Sunshine Coast and Sea World, Gold Coast) and wild (Moreton Bay, Queensland; 27° 25' S; 153° 16' E) environments in order to sample all developmental stages. Captive *C. punctatum* egg cases were removed from breeding tanks on the day of deposition, and tagged with the date. They were then held in continuously filtered and aerated tanks containing natural sea water at ambient temperature. Animal ages were based on hatching age (approximately 153 days post deposition, dpd; Harahush et al., 2007) at a water temperature of 21-25° C. As hatching age altered as water temperature increased (to 27° C) throughout the season, the hatching age used was based on the number of days to hatching of animals at that temperature, converted to a percentage of total development and then equated back to a 153 day embryonic period. Therefore, ages provided should be considered indicative only, as a supplemental comparative tool. Total length (TL) measurements are provided for individuals (where relevant), and otherwise as the average TL for *C. punctatum* embryos used in this study at the particular developmental age. Wild animals were collected with hand or seine nets between dusk and dawn in water less than three metres in depth. Study animals were killed with an overdose of tricaine methane sulfonate (MS222; Sigma, USA) at a ratio of 1:2000 in sea water. All experiments were conducted in accordance with the ethical guidelines set by the

National Health and Medical Research Council of Australia and The University of Queensland Animal Ethics Committee AEC No. ANAT/723/05/ARC.

Assessment of retinal ganglion cell topography

Cell sampling began with 82 dpd embryos (10.1 cm TL), based on the age of the sharks when RGCs first differentiate [Harahush et al., 2009]. A total of 11 embryos (9.6 – 15.2 cm TL), four pups (less than one year old, 14.7 – 25.6 cm TL), two immature juveniles (46.5 – 72.9 cm TL) and five adults (sexually mature, 99.5 – 111.6 cm TL) were examined. Post euthanasia, the eyes were immediately enucleated and the cornea, lens and vitreous were removed. A slit was made in the ventral retina for orientation. Each retina was immersion fixed within the eyecup in 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) for between 30 min and 24 hours, depending on the size of the eye. Fixed retinæ were wholemounted onto gelatinised slides (1.5g gelatine, 7 ml glacial acetic acid, 100 ml 28.5% ethanol, 0.1g chromic potassium, 2 ml dH₂O) and stained with 0.05% cresyl violet for six to twelve minutes, depending on the size of the retina. Outlines were scaled from wholemounted retinæ and measured using Image J (Rasband, W. S., Image J, U. S. National Institutes of Health, Bethesda, USA). Wholemounted retinæ were labelled with an animal code only, so as to not introduce any age-related bias into cell counts. Ganglion cells were identified based on their position within the ganglion cell layer and cell morphology. To minimise the potential for including glial and ‘displaced’ amacrine cells in ganglion cell counts, the size and shape of all three cell types was analysed prior to counting based on criteria according to Collin [1988], where counts of Nissl-stained cells were compared to the number and distribution of ganglion cells retrogradely-labelled from the optic nerve in another species of elasmobranch, *Glaucostegus* [= *Rhinobatus*] *typus*. Briefly, RGCs were characterised on

morphological grounds, as large, granular, irregularly-shaped cells [Collin, 1988]. Amacrine and glial cells, characterised by small, circular, darkly staining cells [Collin, 1988] and elliptical, wispy, filamentous cells, respectively, were not included in the counts. Retinae were viewed at 400x magnification, and cells within a 240 μm x 240 μm (10x10) graticule were manually counted by a single assessor every 0.5 mm for retinae less than 300 mm^2 in area and every 1.0 mm for retinae over 300 mm^2 in area (although areas of higher cell density were sampled more often). Actual cell counts were then converted to cells mm^{-2} . To create the topography maps, iso-density contours were created by joining retinal regions of equal density set at intervals of 250 cells mm^{-2} . Topographic maps were created using Freehand (Adobe Systems Incorporated, San Jose, USA). Total cell counts were calculated by multiplying the average cell density of each retinal contour by its area and adding all areas together.

Assessment of retinal apoptosis

Various methods were attempted to quantify apoptotic cells. Multiple TUNEL and Annexin V protocols were trialled to label apoptotic cells [Harahush, 2009] but unfortunately none of these protocols were successful in this species. During these trials, teleost and mouse retinal tissue, as well as positive controls, were run simultaneously, all resulting in positively stained cells. Therefore, apoptotic cells in *C. punctatum* were differentiated and counted using morphological criteria [Young, 1984, Dunlop and Beazley, 1987, Dunlop et al., 1987, Wong and Hughes, 1987b, Robinson, 1988, Harman and Beazley, 1989]. The ganglion cell layer of stained, wholemounted retina (prepared as described above) were sampled at 0.5 mm or 1.0 mm intervals for small, circular, condensed, darkly-staining cells signifying an apoptotic cell body. All cells matching these criteria within the ganglion cell layer were counted. Although

this method may have inadvertently included apoptosed glial and ‘displaced’ amacrine cells, the level of apoptosis in these two cell populations is thought to be minimal and therefore not contributing appreciably to total counts within the ganglion cell layer or topography [Beazley et al., 1988]. Estimates of apoptotic cell numbers were calculated by dividing the total number of apoptosed cells counted by the total area counted and multiplying that number by the total retinal area.

Calculations of spatial resolving power

One eye from each of eight individuals (embryonic through adult stages, ranging from 15.1 to 95.1 cm TL) was examined for focal length and calculations of spatial resolving power. The eye was removed from the head and frozen in Tissue-Tek O.C.T Compound (Sakura Finetek Europe B. V., Zoeterwoude, The Netherlands). Eyes were serially sectioned equatorially at -25 °C on a Leitz 1720 Digital MGW Lauda Kryostat (Leitz, Wetzlar, Germany) at 50 µm intervals in embryos and pups and 100 µm intervals in juveniles and adults. Digital photographs of the embedded eye were taken every 5th slice, or every 250 µm and 500 µm in embryos/pups and juveniles/adults, respectively, using a digital camera (Sony Cyber Shot, Sony Corporation, Tokyo, Japan). A ruler was placed flush against the block containing the eye in every photograph to provide calibration in mm. The photograph showing the thickest lens diameter was chosen to represent the geometric (visual) axis of the eye. Measurements were made of the axial diameter of the eye and lens and the posterior nodal distance (PND) using Image J (<http://rsb.info.nih.gov/ij/>). These values were used to form the PND:lens radius ratio, often referred to as Matthiessen’s Ratio [Matthiessen, 1880, 1882].

The theoretical spatial resolving power was calculated based on a hexagonal sampling array using the formulae of Snyder & Miller [1977] and Hart [2002]. The distance (d) subtended by one degree of retina was calculated by:

$$d = (2\pi\text{PND}) / 360.$$

The distance between cells (S) was then taken into account using the formula:

$$S^2 = 2 / (D\sqrt{3}),$$

where D is the peak ganglion cell density (cells per mm^2). The maximum spatial frequency (ν) using sinusoidal grating was then calculated by:

$$\nu = 1 / (S\sqrt{3}).$$

This value was then multiplied by d to give a resolution in cycles per degree.

Results

Retinal topography

The eyes of *C. punctatum* are located laterally in the head, with little change in position throughout development (Fig. 1).

During embryogenesis, the distribution of cells within the RGC layer shows pronounced variability (Figs. 2, 3). At the developmental stage of ganglion cell differentiation (82 dpd; 10.1 cm TL), average ganglion cell counts are as low as $285 \text{ cells mm}^{-2}$ (Fig. 4A; Table 1), with peak ganglion cell counts reaching $1,007 \text{ cells mm}^{-2}$ in the centro-nasal part of the retina

(Fig. 4B; Table 1). Densities of cells are almost uniform across the retina, although a narrow strip of increased density oriented obliquely across the equatorial meridian is distinguishable (Fig. 3A; Table 1). By 110 dpd (14.7 cm TL), the distribution of cells has changed with the formation of a wide horizontal streak (mean cell density of around 3,000 cells mm⁻²) oriented across the retinal meridian (Fig. 3B, 4A; Table 1).

By 115 dpd (13.9 cm TL), a weak horizontal streak is present and up to three *areae* (2,500 to 4,250 cells mm⁻²) have developed, with the peak area in the dorsal retina and others in temporal and nasal retina (Fig. 3C, 4B, Table 1). At hatching (16.7 cm TL), topography does not take on any definable pattern, with high densities of cells occupying the majority of the dorsal half of the retina (Fig. 3D). Average ganglion cell density (~3,175 cells mm⁻², Fig. 4A, Table 1) peaks at this stage in development and the peak cell density reaches nearly 5,000 cells mm⁻² (Fig. 4B; Table 1).

Shortly after hatching (24.7 cm TL), the streak becomes more defined. Additionally, several *areae* of increased cell density have formed within the streak (Table 1). A horizontal streak is always present in this species, however, the 'streak' could be more appropriately defined as a horizontal, non-contiguous string of elevated density *areae* across the dorsal hemiretina in juvenile and adult sharks (46.5 – 111.6 cm TL; Fig. 3E, F). Average ganglion cell density reaches its lifelong minimum (post ganglion cell differentiation) during juvenile stages, dropping down to 743 cells mm⁻² (Fig. 4A; Table 1). Peak ganglion cell density also falls to 1,493 cells mm⁻² in juveniles (Fig. 4B; Table 1).

Retinal area increases continuously with TL and ranges from 7.8 mm² in an 82 dpd embryo (9.6 cm TL) to 490.1 mm² in an adult (99.5 cm TL), a relationship which is best fit with a

second order polynomial curve ($y = 0.0039x^2 + 4.2545x - 37.806$; Fig. 4C). Likewise, ganglion cell counts increase with TL from 3,386 cells in an 82 dpd embryo (10.6 cm TL) to 547,881 cells in an adult (99.5 cm TL), a relationship also best fit by a second order polynomial curve ($y = 11.425x^2 + 3083.7x - 16941$; Fig. 4D).

Retinal apoptosis

Cell death in the RGC layer is highly variable in the embryonic retina of *C. punctatum* (Fig. 5), but is almost non-existent in hatched sharks (Fig. 6, 7; Table 1). During embryogenesis, peak density counts range from 33.3 cells mm^{-2} to 0.4 cells mm^{-2} at 82 dpd (9.6 cm TL) and 100 dpd (13.7 cm TL), respectively. Three peaks in apoptosed cell density can be seen in *C. punctatum* (Fig. 7); the first around 82 dpd (9.6 cm TL), the second at 115 dpd (12.5 cm TL) and the third at 140 dpd (16.2 cm TL). Higher RGC apoptosis densities observed during late embryonic stages are maintained until shortly after hatching. Apoptosed RGC densities reach 6.5 cells mm^{-2} in newly-hatched sharks (less than 24 hours old, 18.6 cm TL), but drop to 1.3 – 2.5 cells mm^{-2} in juveniles and adults (46.5 – 111.6 cm TL). Apoptosed cells rarely comprise more than 0.35% of the total cell population, however one animal at 82 dpd, showed an apoptosed cell population equating to 2.0% of the live cell population. The topography of the apoptosed cell population is as variable as the live cell population during embryogenesis, suggesting extremely rapid cycles of cell birth and programmed cell death (Fig. 6). During the periods of elevated retinal apoptosis, the topography of apoptosed cells is generally relatively uniform. However, during the final ~10% of embryonic development (140 dpd, 16.2 cm TL), increased *areae* of ganglion cell apoptosis appear in the ventral retina, distinctly ventral to the developing mid-dorsal horizontal streak seen just after hatching. At other times, apoptosed cells are found primarily in retinal areas corresponding to

regions outside of the high density streak of live cells (Fig. 6), or were so sparse that no marked increases were distinguishable at any retinal location (Fig. 6).

Lens growth and spatial resolving power

The posterior nodal distance (PND) increases from 2.7 mm in embryos (15.1 cm TL, 115 dpd) to 10.0 mm in adult sharks (95.1 cm TL; Table 2). The ratio of the PND to lens radius decreases with growth from a maximum of 4.00:1 in an embryo (18.2 cm TL, 150 dpd) to 2.64:1 in an adult (95.1 cm TL; Table 2). Spatial resolving power (SRP) is lowest in embryonic sharks just prior to hatching (18.2 cm TL, 150 dpd) at 1.47 cycles degree⁻¹, increasing to 4.29 cycles degree⁻¹ in adult sharks (95.1 cm TL).

Discussion

Continuous neural development has resounding effects on an animals' ability to adapt its sensory systems in response to ontogenetic shifts in diet or ecology. The retina of *C. punctatum* shows continuous cell division throughout life, and shows the ability to change (and improve) its visual potential based on environmental and nutritional needs. This provides the animal with precise sensory stimulation at all life stages. Ontogenetic changes in the physiology of the elasmobranch visual system have gone largely unexamined, potentially leading to erroneous conclusions on sensory capability, based on a single data point in a continuously evolving system. This study now presents an ontogenetic timeline of RGC topography, from the earliest stages of retinal cell development during embryogenesis through adulthood.

Development of retinal topography

While the majority of changes in *C. punctatum* occur during embryogenesis, this species clearly shows an alteration in the distribution of its RGCs throughout life. At hatching, *C. punctatum* has a well-defined horizontal streak with several smaller elevated density *areae* within the streak. As the animal and retina continue to grow, the horizontal streak morphs into a non-contiguous string of elevated density *areae*. The ontogenetic changes seen in *C. punctatum* are minor compared to some other anamniotic vertebrates, such as the black bream, *Acanthopagrus butcheri* [Shand et al., 2000a], the European hake, *Merluccius merluccius* [Bozzano and Catalan, 2002] and the tree frog *Hyla raniceps* [Bousfield and Pessoa, 1980]. In most anamniotes, topographic changes in ganglion cell density are generally attributed to changes in feeding behaviour or habitat shifts [Shand et al., 2000a, Shand et al., 2000b]. As it is not expected that *C. punctatum* shows marked shifts in habitat over development, the shift from high density *areae* to a horizontal streak more likely reflects a change in predatory strategy towards larger, more mobile prey.

Factors affecting the development of retinal topography

Our findings suggest that multiple processes affect the changing RGC topography in *C. punctatum*. Total ganglion cell counts increase throughout life, suggesting continuous cell addition. Bromodeoxyuridine (BrdU) studies were attempted to identify and localise newly dividing cells [Harahush, 2009], however, similar to TUNEL and Annexin V trials, we were unable to consistently achieve convincing and reproducible labelling in this species. The other process commonly reported to effect retinal topography is programmed cell death. Retinal apoptosis has been attributed to topographical changes in some vertebrates based on a

smaller proportion of apoptosed cells occurring in the future high density *areae* than elsewhere in the retina and particularly elevated densities of apoptosed cells in the retinal periphery [Dunlop and Beazley, 1987, Dunlop et al., 1987, Beazley et al., 1989].

The distribution of apoptosed cells is non-uniform in embryonic *C. punctatum* retinae; however areas of increased apoptosis occur predominantly outside of ganglion cell high density *areae* suggesting that during these stages, programmed cell death is a factor in defining high density *areae*. The importance of apoptosis in defining RGC topography is variable, even amongst anamniotes. Apoptosis helps to shape the ganglion cell topography of the teleost *Acanthopagrus butcheri* [Shand et al., 2000a, Shand et al., 2000b], but is only a factor in the earliest stages of gestation in amphibians, and not during times of major topographic alterations [Glücksmann, 1940, Beazley et al., 1989]. Only basal levels of apoptotic cells were observed in hatched *C. punctatum*. It is therefore likely that differential cell addition is predominantly responsible for the minor changes in the ganglion cell topography observed between recently hatched sharks and mature individuals.

It is of note that many traditional techniques for assessing cell differentiation and apoptosis were unsuccessful in this study. This presents the question of how selachian (and possibly chondrichthyan) ganglion cell processes and/or structure vary to other vertebrates, and would be an interesting topic for future investigation.

The timing and level of retinal apoptosis

C. punctatum appears to show three peaks in RGC apoptosis at approximately 82 dpd, 115 dpd and 140 dpd, ages which correspond with the differentiation of the first retinal neurons

(mainly ganglion cells), photoreceptor differentiation and synaptogenesis between photoreceptors and their post-synaptic contacts [Harahush et al., 2009]. This suggests that retinal apoptosis in *C. punctatum* is in response to clearing surplus cells that failed to reach and join the optic nerve, and subsequently failed to form synapses within the retina and optic tectum or other central targets [Cole and Ross, 2001, Francisco-Morcillo et al., 2004]. These developmental time points are similar to those seen in the brown trout, *Salmo trutta fario* [Candal et al., 2005]. *C. punctatum* had very little retinal apoptosis post-hatching, similar to other anamniotes [Glücksman, 1940, Beazley et al., 1989, Hoke and Fernald, 1998, Julian et al., 1998, Candal et al., 2005], a trend that has been attributed to better control of the production of cells at lower rates of addition in juvenile and adult teleost fishes [Hoke and Fernald, 1998].

The peak proportion of apoptotic RGCs compared to live cells in *C. punctatum* at the time of RGC differentiation (2.0%, Table 1) is higher than that observed in other vertebrates [Perry et al., 1983, Beazley et al., 1989, Biehlmaier et al., 2001]. Otherwise, the percentage of apoptosed cells in *C. punctatum* (<0.35%; Table 1) is similar to the rates observed in embryonic mammals [Perry et al., 1983, Beazley et al., 1989] and zebrafishes [Biehlmaier et al., 2001]. Depending on the technique used to characterise these cells, the percentage of apoptotic cells in the RGC layer is thought to be severely underestimated due to phagocytosis and clearance of dead cells occurring within just a few hours [Hume et al., 1983, Linden et al., 1986, Pearson et al., 1993]. Perry et al. [1983] reported that during postnatal retinal development in the rat, the loss of over half of the ganglion cells is accompanied by the instantaneous detection of less than 1% of degenerating profiles at individual time points. It is thought that amniotic vertebrates have a significantly greater amount of RGC apoptosis compared to anamniotic vertebrates, with retinal apoptosis in anamniotes more restricted to

‘fine tuning’ the neuronal network than eliminating large numbers of cells, which failed to connect to their targets [Biehlmaier et al., 2001]. Since anamniotes continually increase their eye size, they may reserve the majority of their generated cells for future growth, thus saving the energy required to remove cells, only to later generate more [Biehlmaier et al., 2001].

Retinal surface area and ganglion cell counts

As seen in other anamniotes [Lyll, 1957, Johns, 1977, Beazley et al., 1989, Fernald, 1991, Bozzano and Catalan, 2002], retinal surface area in *C. punctatum* increases with animal total length. Similarly, total live ganglion cell counts increase linearly with the total length of the animal. Ganglion cell counts in *C. punctatum* peaked at 547,881 cells in a 99.5 cm TL adult. While this was the highest cell count observed in this study, it is possible that total numbers could be even greater, as this species can reach up to 125 cm TL in the wild and 150 cm TL in captivity [Harahush et al., 2007]. Despite attempts to morphologically discriminate and differentially count the different cell types within the ganglion cell layer of *C. punctatum*, some amacrine cells may have been inadvertently included in cell counts. The percentage of ‘displaced’ amacrine cells in the ganglion cell layer varies by species, ranging from 5% in turtles [Peterson and Ulinski, 1979] to over 80% in certain deep sea teleosts [Wagner et al., 1998] and some mammals (i.e. cats, Wong and Hughes, 1987a). Using retrograde labelling from the optic nerve, Lisney & Collin [2008] found that approximately 76% of the cells in the ganglion cell layer of the juvenile *C. punctatum* central retina were non-ganglion cells. The influence of these cells on the ganglion cell topography, however, was not assessed. The topographic distribution of RGCs and peak cell densities often remain relatively unchanged, despite the inclusion of ‘displaced’ amacrine cells and other non-ganglion cells [Collin, 1988, Collin and Pettigrew, 1988, Pettigrew et al., 1988, Collin, 1999], but total cell population

counts have the potential to be grossly overestimated. However, ‘displaced’ ganglion cells out of focal range were not included in cell counts in the present study, introducing scope for underestimation of the total ganglion cells within the retina.

Visual acuity

Current results show an increase in the PND to lens ratio over mid-to-late embryogenesis in *C. punctatum*, which is opposite to the decreasing trend expected. The increase appears to be more related to variation in lens diameter between animals, as opposed to eye diameter, which shows steady increases over our developmental series. This may therefore suggest that the development of the lens is not highly regulated immediately prior to and during this stage of development. As *C. punctatum* egg cases are still semi-opaque during the latter stages of embryonic development, the effects of varying growth rates between the eye and lens would have little impact on visual capability. Rather, it is thought that light/ shadow sensing capabilities may instead be the visual focus at this stage [Unpublished observations].

The ratio of the PND to the lens radius (often referred to as Matthiessen’s ratio; Matthiessen, 1880, 1882) in *C. punctatum* decreases with growth. *C. punctatum* shows an ontogenetic drop in this ratio, from 4.00:1 in late embryogenesis to 2.63:1 in adults. Post-hatching decreases in Matthiessen’s ratio during development are thought to compensate for the constraints imposed by a small eye in young fish [Shand et al., 1999]. Similar decreases have also been seen in the clearnose skate, *Raja eglanteria* [Sivak and Luer, 1991] and the black bream, *Acanthopagrus butcheri* [Shand et al., 1999]. *R. eglanteria* shows a much smaller range (2.75:1 in pups to 2.55:1 in adults), whereas the bream has a similar range (4.00:1 to 2.41:1) to *C. punctatum*. However, the decrease in bream PND occurs rapidly over the first 20 days

of life, as opposed to the steady lifelong decrease in *C. punctatum*. Although the peak ganglion cell density in *C. punctatum* decreases from embryogenesis/ hatching to juvenile and adult stages, the spatial resolving power (SRP) increases nearly three-fold between late embryogenesis (1.47 cycles degree⁻¹) and adult stages (4.29 cycles degree⁻¹) due to the continuous growth of the lens and increasing focal length. This increase is contrary to ontogenetic decreases in visual acuity in amniotes [Pitts, 1982, Hodos et al., 1991, Schmid and Wildsoet, 1998] due to their static post-developmental lens size. Lower PND to lens radius ratio signifies heightened retinal illumination and sensitivity [McFarland, 1991] and the increase in SRP would enable the shark to sense more mobile prey at a greater distance.

The developing visual system of C. punctatum in relation to life history and ecology

Although there is very little documented information on the ontogenetic ecology and prey preferences of *C. punctatum*, the findings of this study suggest that while the visual system has the potential to show dramatic ontogenetic changes, RGC topography does not show significant variation. The increase in SRP combined with the separation of high RGC density *areae* within/ from the horizontal streak, however, suggests that *C. punctatum* may shift to more active, focused predation tactics providing a greater variety of prey. This could result in increased nutritional benefits needed for growth and reproduction. The breakdown in the uniformity of the streak could also represent a shift in behaviour, from one of utmost importance in continually scanning the horizon for predators at the highly vulnerable pup stage, to a subsequent investment in predation.

Acknowledgements

We would like to thank the managers, curators and aquarists at UnderWater World and Sea World for their kind donation of time and captive animals, which made this study possible. Thanks also go to J. Page for his efforts and assistance with the collection of wild animals. We also thank C. Bartlett and S. Dunlop for their discussions on, and assistance with qualifying and quantifying retinal apoptosis. This research was funded by an Australian Research Council Discovery Grant to SPC.

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Table 1. Normal and apoptosed ganglion cell counts and topographic patterns of cell distribution in *Chiloscyllium punctatum* over the various life stages sampled. Live ganglion cells (GC) were determined by morphology, staining and retinal location, whereas apoptosed cell counts include all apoptosed cells in the ganglion cell layer, as cell morphology could not be discerned. a, high density area; c, central; d, dorsal; dpd, days post deposition, dph, days post hatch; ds, diagonal streak; hatch, hatchling; hs, horizontal streak; juv, juvenile; n, nasal; r, random; t, temporal; u, uniform; v, ventral. – Apoptosed cell analysis could not be conducted on the 140 dpd retina due to tissue degradation.

Age	TL (cm)	Live cells				Apoptosed cells			
		Topography	Total GC count	Peak density (cells mm ⁻²)	Average density (cells mm ⁻²)	Location	Total GC count	Average density (cells mm ⁻²)	% of apoptosed cells
82 dpd	9.6	r/ ds	12875	3177	1656	u	259	33.3	2.00
82 dpd	10.6	ds	3386	1007	285	u	0	0.0	0.00
100 dpd	13.7	hs (c)	53211	3316	2168	r	10	0.4	0.02
110 dpd	14.3	r/ hs (c)	83813	4288	3040	r	54	2.0	0.06
110 dpd	15.1	hs (c)	55430	4444	2838	v	31	1.6	0.06
115 dpd	12.5	r	73832	4063	2889	d,t	178	7.0	0.24
115 dpd	15.2	3a (d,n,t)/hs (c)	67717	3733	2365	v	60	2.1	0.09
124 dpd	15.2	hs (d)	102580	4826	3018	v	155	4.6	0.15
124 dpd	16.2	r	102601	4392	3194	v	345	10.8	0.34
140 dpd	15.2	r	94026	3889	2582	-	-	-	-
150 dpd	16.2	hs (n)	60859	2951	1857	t	144	4.4	0.24
1 dph	14.7	r	73761	4167	3122	v	91	3.9	0.12
1 dph	18.6	r	60419	4983	3228	u	122	6.5	0.2
Hatch	23.8	hs (d)	95330	2274	1390	u	67	1.0	0.07
Hatch	25.6	3a/ hs (d)	98951	2465	1296	u	130	1.7	0.13

Juv	46.5	lose hs (d)	199031	3212	1560	v	199	1.6	0.10
Juv	72.9	hs (d)	226396	1493	743	v	359	1.3	0.17
Adult	99.5	3a (c,t)/ hs (d)	547881	2222	1118	u	1225	2.5	0.22
Adult	111.6	1a (t)/ hs (d)	444559	2604	1022	u	550	1.3	0.12

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Table 2. Ocular measures of eye and lens diameter in the axial plane, posterior nodal distance (PND) and the ratio between PND and lens radius of embryonic through adult stages of *Chiloscyllium punctatum*. Spatial resolving power (SRP) is calculated from the peak ganglion cell density assuming a hexagonal mosaic.

TL (cm)	Class	Lens diameter (mm)	Eye diameter (mm)	PND (mm)	PND: Lens radius	SRP (cycles degree ⁻¹)
15.1	embryo	2.10	4.05	2.70	2.57:1	1.74
15.2	embryo	1.85	4.35	3.0	3.24:1	1.53
18.2	embryo	1.85	5.25	3.70	4.00:1	1.47
61.6	juvenile	5.90	13.25	8.50	2.88:1	2.73
72.6	juvenile	6.60	15.10	9.40	2.85:1	3.05
80.0	adult	7.00	15.35	9.65	2.76:1	3.23
81.9	adult	6.60	15.25	9.67	2.93:1	3.06
95.1	adult	7.60	16.20	10.03	2.64:1	4.29

689 **Figure 1.** The head and eye of *Chiloscyllium punctatum* over development. A-B, 52 dpd; C-
 690 D, 124 dpd; E-F, pup; G-H, adult. Scale bars, A, 1 mm; B, 2 mm; C, 1.5 cm; D, 1 mm; E, 1
 691 cm; F, 1 mm; G, 10 cm; H, 10 mm.

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693 **Figure 2.** Areas of high (A, C, E, G) and low (B, D, F, H) retinal ganglion cell density in
 694 embryonic (110 dpd, 14.3 cm TL; A, B), hatchling (18.6 cm TL; C, D), juvenile (46.5 cm TL;
 695 E, F) and adult (99.5 cm TL; G, H) *Chiloscyllium punctatum*. Arrows show examples of the
 696 morphological and staining quality of the ‘live’ cells counted. Chevrons show the type of
 697 cells (glia) that were not included in ‘live’ cell counts. Scale bars, 30 μ m.

698

699 **Figure 3.** Changes in retinal topography of ganglion cells in *Chiloscyllium punctatum*. Note
 700 the numerous changes during embryogenesis (A, 82 dpd, 10.6 cm TL; B, 110 dpd, 14.3 cm
 701 TL; C, 115 dpd, 15.2 cm TL) after which a thick horizontal streak pattern forms at hatching
 702 (18.6 cm TL, D). The streak becomes much thinner after hatching (25.6 cm TL, E) and is
 703 eventually formed by multiple *areae* in juvenile and adult (111.6 cm TL, F) stages. Black
 704 dots with white outline represent the optic nerve head. N, nasal; V, ventral. Scale bars, A-D, 1
 705 mm; E, 2 mm; F, 4 mm.

706

707 **Figure 4.** Average (A) and peak (B) ganglion cell densities (per mm^{-2}) during development.
 708 Note densities are highest just prior to and just after hatching (left and right to dotted line).
 709 Cell densities then decrease rapidly, and level out for the remainder of life. Conversely,
 710 retinal area (C) and total ganglion cell number (D) increase during embryogenesis and
 711 throughout life, where these relationships are best fit by second order polynomials. There is a
 712 small spike in total ganglion cell number during the last month of embryogenesis, however,

by juvenile stages, cell counts have exceeded those during the embryonic spike. Dotted lines represent the average hatching age of 19.2 cm TL for this species at 22 - 25 °C [Harahush et al., 2007], but are not necessarily the hatching age of individuals in this study.

Figure 5. Representative examples of apoptosed cells (arrows) in the retinal ganglion cell layer of *Chiloscyllium punctatum* during embryogenesis (110 dpd, 14.3 cm TL, A), at hatching (18.6 cm TL, B) and in juvenile (46.5 cm TL, C) and adult sharks (99.5 cm TL, D). Scale bars, 20 µm.

Figure 6. Retinal apoptosis in the ganglion cell layer of *Chiloscyllium punctatum* over development. Rates of apoptosis are highest during embryogenesis (A, 82 dpd, 10.6 cm TL; B, 115 dpd, 12.5 cm TL; C, 140 dpd, 16.2 cm TL) with little or no apoptosis in the developing horizontal streak during mid-embryogenesis (A, B) (see inset for the retinal ganglion cell topography of the same eye, A-D, F, and an eye from the same developmental stage, E). Retinal ganglion cell apoptosis increases during late embryogenesis (140 dpd, 16.2 cm TL, C), but decreases somewhat by hatching (18.6 cm TL, D). Therefore, it is believed that retinal apoptosis plays a significant role in the development of the horizontal streak pattern seen in hatched *C. punctatum*. After hatching (E, juvenile, 25.6 cm TL; F, adult, 111.6 cm TL), apoptosed cells are found uniformly throughout the retina and at basal levels. Blue dots represent the optic nerve head. N, nasal; V, ventral. Scale bars, A-D, 1 mm; E, 2 mm; F, 4 mm. See Figure 3 for the inset figures colour scale. Additional yellow scale has been added to B and C for further clarity in differentiating densities from 2500 cells mm⁻² (light yellow) to 5000 cells mm⁻² (beige).

Figure 7. Retinal apoptosis counts in the ganglion cell layer. Retinal apoptosis is highest during embryogenesis (A, inset), but then drops off to basal levels just after hatching, remaining low for the remainder of life (A). It appears that *Chiloscyllium punctatum* undergoes three peaks of increased retinal ganglion cell apoptosis at 82 dpd (9.6 cm TL), 115 dpd (12.5 cm TL) and 140 dpd (16.2 cm TL), which correspond to the times of ganglion cell differentiation, photoreceptor cell differentiation and just after the formation of synaptic connections. The percentage of apoptosed cells in the ganglion cell layer compared to the live cell population (B) shows a similar elevated trend during embryogenesis, falling to basal levels of around 0.2% in hatched sharks of all ages. Dotted lines represent the average total length at hatching (19.2 cm) for this species at 22 - 25 °C [Harahush et al., 2007], but are not necessarily the hatching age of individuals in this study.