THE ROLE OF NEUROMASTS IN NON-VISUAL FEEDING OF LARVAL STRIPED BASS (*Morone saxatilis*)

by

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Dedication

This thesis is dedicated to my husband, Dave, for all of his love, support, and encouragement.

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Abstract

Striped bass larvae, native to the Shubenacadie River, catch invertebrates in darkness using mechanoreception via lateral line neuromasts. The neuromast total increased from 17 at first feeding (5 to 7 dph) to 135 by the juvenile stage (27 dph). A 5 mM neomycin dose ablated neuromasts, confirmed by fluorescent and confocal microscopy. In feeding trials, larvae with and without functional neuromasts were offered *Artemia salina* in darkness or light. To identify ontogenetic changes in feeding, experiments were repeated at 10, 13, 17, and 20 dph. In darkness, neomycin treated larvae caught fewer prey (~5 *Artemia* h^{-1} at all ages, $p<0.05$) than larvae with intact neuromasts (10 dph, 16 *Artemia* h^{-1}; 20 dph, 72 *Artemia* h^{-1}). In light, neomycin did not affect feeding, indicating no deleterious side-effects. Neomycin did not damage olfactory or taste cells judged by FM1-43FX and calretinin staining. The results support the contribution of mechanoreception to non-visual feeding.

Acknowledgements

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Chapter 1 Introduction

Mortality is generally high for early stage larval fish (Houde, 1989). The survival of larval fish is largely dependent on feeding capability, but early stage larvae are generally poor feeders, at least until fins and vision become better developed (Krebs and Turingan, 2003; Wanzenbock and Scheimer, 1989). Marine fish larvae inhabiting clear oceanic waters are primarily visual feeders and require a functional retina for the onset of first-feeding (Hunter, 1981; Blaxter, 1986). As such, existing literature on larval feeding mechanisms is mainly focused on visual feeding. However, estuaries which are turbid in nature, such as the Shubenacadie-Stewiacke River, may not be conducive to visual feeding. Striped bass (*Morone saxatilis*), in particular, are the focus of study for this thesis as they are successful inhabitants of the Shubenacadie River.

In Atlantic Canada, nine estuaries once supported striped bass breeding populations including three in the Bay of Fundy: the Annapolis, the Saint John, and the Shubenacadie Rivers (Rulifson and Dadswell, 1995). However, two spawning populations have since been lost. The Mactaquac Dam was built on the Saint John River in the 1960's which impeded fish passage and increased sedimentation resulting in decreased water quality (Wells, 1999). On the Annapolis River, a causeway dam was reconstructed in 1984 and a tidal power generating station was built which again reduced passage of striped bass and increased sedimentation (Wells, 1999).

The Shubenacadie-Stewiacke River, which is dominated by a tidal bore, is the only remaining estuary for striped bass spawning populations in the Bay of Fundy (Rulifson and Tull, 1999). As a result, this population of striped bass has been classified as a threatened species by the Committee on the Status of Endangered Species (COSEWIC) in their 2004 assessment (COSEWIC, 2004). Non-visual feeding may be used by larval striped bass inhabiting this turbid estuary, but relatively little data exists on the subject (Chesney, 1989).

The decline in striped bass spawning populations in Atlantic Canada is cause for concern. However, despite the loss of these striped bass spawning populations and high larval mortality, the numbers of striped bass have increased in the Bay of Fundy due to

the remaining Shubenacadie-Stewiacke River population which continues to reproduce successfully (DFO, 2006).

Feeding success at early larval stages is related to factors such as recruitment and population dynamics. During the first year, over winter survival is dependent on body size as there is evidence that young-of-the-year must reach a critical length of 10 cm (Kenneth and Cowan, 1993; Hurst and Conover, 1998). The need to grow as fast as possible to reach a specific weight before winter creates strong selective pressure to maximize feeding throughout the night (Hurst and Conover, 1998). However, the ability of striped bass larvae to feed in the highly turbid Shubenacadie River during the day is a challenge in itself. Non-visual feeding in both dark and turbid environments has been observed for striped bass larvae (McHugh and Heidinger, 1977; Chesney, 1989). Thus, the question that is presented is how the larvae, primarily visual feeders, are effectively finding prey.

Mechanoreception via lateral line neuromasts could potentially be involved in larval non-visual feeding (Mukai, 2006). One prey capture study on striped bass larvae using streptomycin, an antibiotic which damages neuromasts, found that feeding was reduced in the dark, but prey capture was low even for controls making the results inconclusive (MacIntosh, 2006). The role of neuromasts in non-visual feeding has potential, but the results to date provide weak evidence partly due to the lack of quantification of neuromast damage, but also the lack of knowledge on neuromast ontogeny for striped bass. Thus, it is currently unknown whether striped bass larvae rely on neuromasts for non-visual feeding. The objective of this investigation is to understand how early-stage striped bass larvae are able to survive in turbid environments by examining the mechanism of non-visual feeding.

1.1 Status and Life Cycle of Striped Bass in Atlantic Canada

The sexual maturation of striped bass begins in the fall and is associated with most adult striped bass, ranging in age from 2 to 13 years old, entering Grand Lake, N.S. to spend the winter (Rulifson and Dadswell, 1995). In May, adults migrate down the Shubenacadie River to spawn in the Stewiacke River when temperatures average around 16°C (Coutant, 1990; Rulifson and Dadswell, 1995). Adults then return to the ocean for

the summer, some migrating down the coast to U.S.A. waters (Rulifson and Dadswell, 1995). Fertilized eggs are semi-buoyant and are maintained in suspension by the turbulent current (Rulifson and Tull, 1999).

Striped bass larvae normally have high mortality, which is typical of pelagic fish, and are only 4 mm in length at time of hatching (Eldridge *et al.*, 1982; Houde, 1989). The eggs hatch within 48 hours and between 5 and 10 dph, the larvae begin to feed as the yolk sac becomes absorbed (Secor and Houde, 1995; MacIntosh and Duston, 2007). Spawning takes place over a four week period and all experimental work had to be completed within this short time period. The three stages of larval development include the yolk sac stage which lasts for 7-14 days when larvae are between 5 and 8 mm long, the fin fold stage (8-12 mm) which lasts for 10-13 days, and the post fin fold stage when larvae reach >25 mm around 30 dph (Doroshev, 1970; Polgar *et al.*, 1976; Mihursky *et al.*, 1976). At the post fin fold stage (~30 dph), the fish are considered juveniles as this is when the fins are fully formed and the external morphology is similar to the adult (Hardy, 1978). After 1-2 years, young-of-the-year migrate with the adults from the Shubenacadie River to Cobequid Bay to continue feeding and growing (Rulifson and Dadswell, 1995).

In large estuaries, such as the Chesapeake Bay, the major nursery habitat for U.S.A striped bass is the area of highest turbidity known as the estuarine turbidity maximum (ETM; North and Houde, 2003). The ETM provides higher concentrations of zooplankton, protection from visual predators, as well as optimal temperature and salinity conditions (Strathmann, 1982; Chesney, 1989; Simenstad *et al.*, 1994). Larvae reared in extremely turbid conditions, such as the Shubenacadie and the Maryland Rivers, must cope with the challenge of catching prey under conditions where visual feeding may be impossible due to both the absence of light and reduced visual acuity (Chesney, 1989).

Light is commonly referred to as radiation in the visible spectrum of 400 to 700 nm. In clear, deep water, red wavelengths are absorbed within the first 2 m and infrared light is absorbed in the first 20 cm, which is then converted to heat energy (Kirk, 1994; Wozniak and Dera, 2007). Light attenuates with depth due to the loss of energy from absorption and scattering with blue and green light penetrating the furthest (McCluney, 1975; Wozniak and Dera, 2007). However, light of shorter wavelengths scatters more easily with blue light scattering 10 times more than red light (Moore *et al.*, 1980).

Scattering is due to the deflection of light by suspended microscopic particles, especially in turbid waters typical of tidal-dominated coastal waters like the Shubenacadie River in Nova Scotia (McCluney, 1975; Heap *et al.*, 2001). Turbidity is defined as a measurement of the intensity of unscattered light transmitted through a medium (McCluney, 1975). In contrast, nephelometry is defined as a measurement of intensity of scattered light usually at 90 degrees to the incident beam of light (McCluney, 1975). Attenuation of light energy with depth is referred to as irradiance and is defined as the energy per unit area of a surface per unit time measured in Watts per meters squared or quanta per seconds per meters squared (Kirk, 1994).

Turbidity, measured as total suspended solids (TSS), is at least 60 mg l^{-1} in the Chesapeake estuary and is up to five times higher (340 mg l^{-1}) for the Shubenacadie estuary (North and Houde, 2001; Jacques Whitford, 2007). In 1999, the downwelling irradiance of the Shubenacadie River was measured by E. Horne, Bedford Institute of Oceanography using a Spectroradiometer (model MER-2048) following a request from J. Duston. Only the first two meters of the river were penetrated by light on a sunny day, with light intensity declining from 800 to 400 lx in the first 50 cm, reaching 140 lx at one meter, and 0 lx at 2 m (Fig. 1.1). Lux is defined as illumination of a lumen per sqare meter. Visibility is low in the Shubenacadie River due to the high turbidity, but turbidity in itself has not always been found to have detrimental effects on prey capture by striped bass larvae (Breitburg, 1988). Striped bass larvae can survive and grow, even with turbulence, high turbidity (150 mg l^{-1} kaolin), and complete darkness (McHugh and Heidinger, 1977; Chesney, 1989). However, these studies have not identified the mechanism by which striped bass larvae feed in the dark and one did not even quantify prey capture in the dark (McHugh and Heidinger, 1977). The most recent study on larval striped bass also reported feeding in the dark, but at a low rate without determining the feeding mechanism (MacIntosh and Duston, 2007). The sensory mechanism these larvae are using to feed in the dark is still unknown.

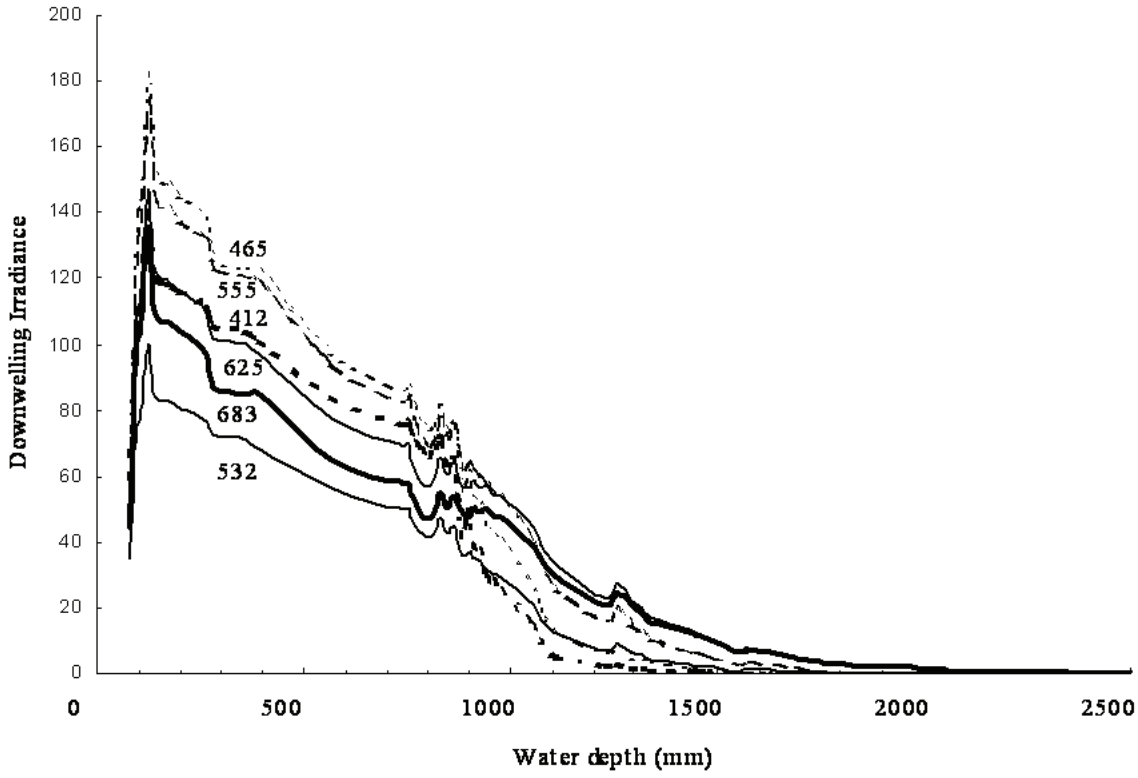

Figure 1.1 Downwelling irradiance in the Shubenacadie River (latitude: 45 15.805, longitude: 63 29.640) at noon on a sunny September day (1999) showing six wavelengths ranging from 412 nm (blue) to 683 nm (red) which penetrate the first 2 m of the water (units: μW cm^{-2}; 1 μW cm^{-2} = 6.83 lux at 555 nm).

1.2 Non-visual Feeding in Fish

Within the animal kingdom, the absence of visual information often results in a reliance on a number of other senses. Red knot sandpipers (*Calidris canutus*) probe wet sand with their bill which contains a pressure sensory organ called the Herbst corpuscle to detect bivalve *Macoma balthica* prey buried up to 5 cm deep (Piersma *et al.*, 1998). Spiders have sensory receptors on their legs called slit sensilla which respond to web vibrations when prey are caught and will even respond to a vibrating tuning fork placed on the web (Bays, 1962; Barth and Geethabali, 1982). The Brazilian yellow scorpion (*Tityus serrulatus*) has pectines which are a pair of comb-like sensory organs situated on the sternum and are constantly in contact with the ground (Root, 1990). These organs are mechanoreceptive in that they detect vibrations of prey short distances away, but this ability is inhibited when the pectines are covered with paraffin wax (Mineo and Claro,

2006). Crocodilians and allegatorids have touch papillae resembling specialized mechanoreceptors, which they are able to line up with the water's surface. The corresponding nerves phase lock with the waves, enabling the crocodile to orient toward disturbances as small as a single drop of water hitting the surface in total darkness (Leitch, 2010). These examples show that visual information is not necessary for prey detection and that certain habitats may result in specific biological adaptations, which in many cases involve mechanoreception.

Non-visual feeding has been reported in a number of fish species as well. Northern anchovy larvae (*Engraulis mordax*), which are primarily visual feeders, often feed as deep as 74 m during the day and only at the surface under moonlight (Bagarinao and Hunter, 1983). However, in lab trials, 10% of northern anchovy larvae (10 to 15 mm total length, TL) could feed in the dark catching between 5 and 32 rotifers (*Brachionus plicatilis*) 1.5 h^{-1} (Bagarinao and Hunter, 1983). The fathead minnow (*Pimephales promelas*) shows preference for live rather than dead *A. salina* in the dark (Salgado and Hoyt, 1996). In the case of striped bass larvae reared in the turbid Shubenacadie-Stewiacke River estuary, light does not reach below the first 2 m on a sunny afternoon (Fig. 1.1; E. Horne, Bedford Institute of Oceanography). Limited light severely reduces visual acuity (Arctic grayling; *Thymallus arcticus*, Schmidt and O'Brien, 1982; large mouth bass; *Micropterus salmoides*, McMahon and Holanov 1995). Therefore, there is an advantage for secondary sensory mechanisms for detecting prey, such as mechanoreception, even within the first 2 m of the water column.

Striped bass larvae were first reported to feed in the dark by McHugh and Heiginger (1977) at 9, 15, and 19 dph for 1 to 8 hours suggesting that night feeding was possible, although prey capture was not quantified in this study and only 10% of the larvae fed. In a study investigating nocturnal feeding, 12 dph striped bass larvae were the most active nocturnal feeders compared to other ages ranging from 7 to 26 dph. All larvae fed nocturnally at limited rates , but when fed a high prey density of 5000 *A. salina* l^{-1}, 12 and 21 dph larvae ate at slower rates during the day and extended feeding into the night (Eldridge *et al.*, 1981). Feeding of striped bass larvae in complete darkness was verified with the use of a light sensor to ensure no light was available during feeding (Chesney, 1989). Striped bass larvae fed a prey density of 100 *Eurytemora affinis* l^{-1} in

complete darkness for 20 days (5 to 25 dph) exhibited 70% survival when compared to 70% of same age larvae surviving at a high light intensity of 1000 lx (Chesney, 1989). These findings established that striped bass larvae are capable of feeding in the dark and use a secondary mechanism for prey detection.

When visual feeding is not possible, prey density becomes an important factor for prey capture. Sea bream hatched and exposed to a prey density of 100 or 500 prey l^{-1} for 16 days exhibited poor survival (2% and 5-8% respectively) with a stocking density of 16 to 32 fish l^{-1} (Houde, 1975). A density of 200 prey l^{-1} lays between these, posing a challenge for survival and requiring larvae to actively search out prey. In the Chesapeake tributary, a prey density range of 25 to 500 prey l^{-1} is available to striped bass larvae, but 100 to 200 prey l^{-1} is average (Beaven and Mihursky, 1979; Setzler-Hamilton *et al.*, 1982). However, prey densities of the wild may be under estimated as larvae may congregate in areas of denser food patches and striped bass larvae fed in laboratories at these low densities (100 to 500 prey l^{-1}) exhibit low survival rates (Eldridge *et al.*, 1981).

In the wild, *Eurytemora affinis* is a common prey item in many North American estuaries (Katona, 1971). *E. affinis* concentrations are highest near the Chesapeake Bay estuarine turbidity maximum (ETM) and as such are an important prey item for striped bass larvae (North and Houde, 2001; Kimmel and Roman, 2004). Of the prey items consumed by striped bass larvae located in the Choptank River, Maryland, an estimated 53% to 99% were *E. affinis* from 1983 to 1985 (Uphoff, 1989). *E. affinis* tend to dominate during the winter and spring season while *Acartia tonsa* dominate during the summer and fall (Kimmel and Roman, 2004). Both of these copepods are similar in size to *Artemia salina*. At the first nauplius stage, *A. salina* measure 0.46 mm in length compared to 0.49 mm for *E. affinis* at the first copepod stage (Katona, 1971) and 0.46 mm for *A. tonsa* at the second copepod stage (Heinle, 1966).

In the lab, prey capture was also possible for striped bass larvae feeding in turbid water (150 ppm kaolin) at 0.4 to 450 lx or complete darkness. The challenge of feeding in turbid conditions occurs at high light intensities (2000 lx) when prey lose contrast against the background as demonstrated in striped bass larvae and in light intensities occurring below 450 lx as demonstrated in larval bluegill (Chesney, 1989; *Lepomis macrochirus*; Miner and Stein, 1993). Visual feeding may then be possible in turbidity when light

intensity ranges between 450 and 2000 lx, but a non-visual mechanism is implemented in low light, turbidity, and complete darkness (Chesney, 1989). Larval white perch (*Morone americana*), a congener of striped bass, were observed feeding in the Chesapeake Bay ETM at subthreshold light levels for visual feeding in m id-depth to bottom waters of the turbid upper Chesapeake Bay and its tributaries (Shoji *et al.*, 2005). This finding further suggests a secondary means of prey detection for larval non-visual feeding.

Visual feeding for striped bass larvae may only be possible within the first 50 cm of the Shubenacadie on a sunny afternoon, as light intensity drops to 400 lx at this depth. In addition, there are seven hours of darkness during the summer months prohibiting visual feeding at night. Without visual information in their darkened, turbid estuaries, striped bass larvae are potentially relying on mechanoreception, audition, olfaction, gustation or a combination of these. I hypothesized that sensory organs referred to as neuromasts, found along the lateral line, are able to detect frequencies emitted by prey, thereby facilitating larval prey capture in darkness via mechanoreception.

a) Mechanoreception

The lateral line of teleost fishes, composed of neuromast mechanoreceptors, plays a role in detection of water currents and prey, predator avoidance, and schooling (Montgomery et al., 1997; Blaxter and Fuiman, 1992; Partridge and Pitcher, 1980). There are two types of neuromasts: superficial and canalicular. Superficial neuromasts mediate rheotactic behaviour in teleosts (torrent fish, *Cheimarrichthys fosteri*; blind cavefish, *Astyanax fasciatus*; Antarctic fish, *Pagothenia borchgrevinki*) which is the orientation of the fish's body upstream (Montgomery et al., 1997). Rheotaxis facilitates prey detection as prey items and scents flow downstream.

The development of canal neuromasts and filling of the otic bulla are associated with improved predator avoidance in larval herring (*Clupea harengus L.*; Blaxter and Fuiman, 1990). Before these two events take place, larval responses to predator attacks are low. The defence strategy of newly hatched and transparent herring and plaice is to remain motionless in the presence of a predator (Batty and Blaxter, 1992). Some fish, such as the Hawaiian anchovy (*Stolephorus purpureus*) and the jack (*Caranx ignobilis*),

form schools to decrease the likelihood of individual fish being attacked by a predator (Major, 1978). Predator fish that school are more effective at dispersing a school of prey and isolating individuals (Major, 1978). Schooling depends on both vision and the lateral line, with vision responsible for distance and angle between neighbouring fish and the lateral line responsible for monitoring velocity and direction of swimming neighbors (Partridge and Pitcher, 1980).

In addition to detecting water currents and aiding in schooling, the lateral line is capable of detecting vibrations of swimming zooplankton prey within short distances (Bleckmann, 1993; Montgomery *et al*., 2002). Neuromasts occur on the head, trunk, and tail, but specific locations and ontogeny vary with species (Fig. 1.2; Blaxter *et al.*, 1983; Harvey et al., 1992; Fuiman *et al.*, 2004). A neuromast contains hair cells, each with a graded arrangement of hairs on the tip consisting of one kinocilium (the tallest hair on the hair cell) and many shorter stereocilia (zebrafish, *Danio rerio*; Ou *et al.*, 2007). The hairs are embedded in the cuticular plate and are covered by a dome-shaped layer of gelatinous substance called the cupula which acts as the receiver for sensory information (Fig. 1.3; Fuiman *et al.*, 2004; Bleckmann *et al.*, 2004). Motion of the cupula results in motion of the fluid inside, causing motion of the kinocilia and stereocilia which are directionally sensitive (Blaxter, 1987). When a source of vibration is present, the hair cells are deflected from the motion of the cupula in the direction which opens the tip-links (van Netten, 1997). Calcium ions flow into the sensory cell causing release of glutamate into the synapse and subsequently changing the firing rate of the afferent nerons at the base of the receptor cells (Flock and Wersall, 1962; Baumann and Roth, 1986; Obholzer *et al.*, 2008). The brain then uses the population activity of the afferent neurons to compute the location of the source (van Netten, 1997; Coombs and Braun, 2000). In a study on eel (*Anguilla anguilla*) neuromasts, mechanosensitivity increased with increasing concentration of calcium in the water outside the apical membrane, while sodium and potassium had no influence (Baumann and Roth, 1986).

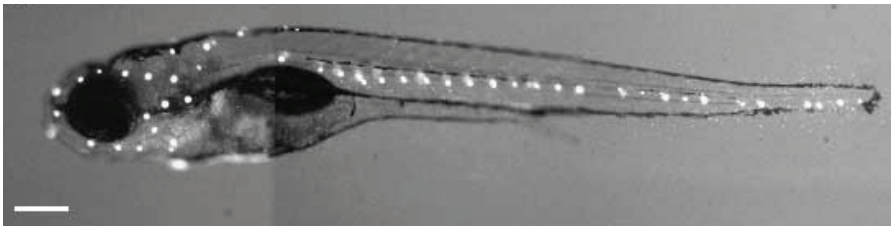

Figure 1.2 Stereotypical positions of neuromasts (white spots) on a 5 days post fertilization (dpf) zebrafish larva stained with (2-(4-dimethylaminostyryl)-N-ethyl pyridinium iodide (DASPEI) and imaged under a fluorescent microscope. Scale bar is 200 μm. Image reproduced with permission (Whitfield *et al.*, 1996).

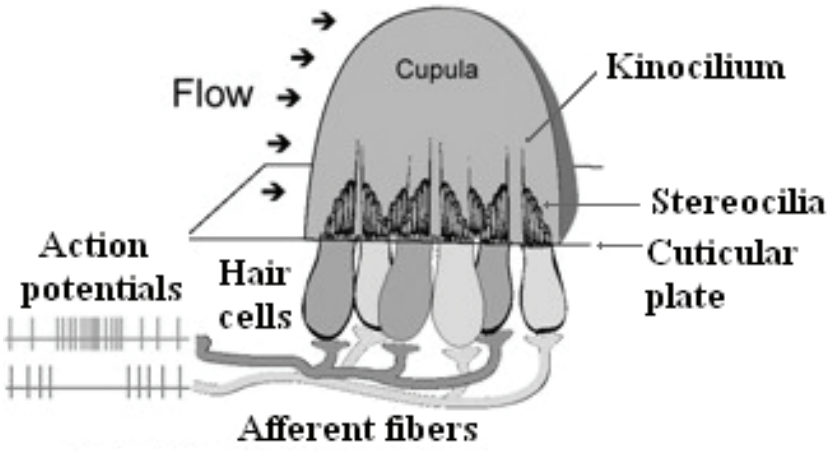

Figure 1.3 Structure of a neuromast cell showing the gelatinous cupula covering the kinocilia (tall) and stereocilia (short) which project from their respective hair cells. The hair cell afferent fibers run toward the brain delivering mechanosensory information via action potentials. The hairs are oriented at 180˚ to each other. Diagram adapted with permission (Bleckmann *et al.*, 2004).

The lateral line nerve was first found to be continuously active at a base rate by Hoagland (1932) in a study on catfish (Siluridae family). The impulses stopped when either the nerve or the neuromasts were anesthetized. Thus, the baseline of impulses provides a background against which the detection of adequate signals is facilitated through deflection of the neuromast hairs (Hoagland, 1932).

Superficial neuromasts emerge through the skin during development, having a cupula which remains directly exposed to the external environment (Jones and Janssen, 1992; McHenry and van Netten, 2007). The hair cells of a neuromast are similar to the hair cells of the otolithic organ and are located centrally within a neuromast, while support cells encompassing the perimeter, secrete the cupula, and are thought to proliferate and replace dying central hair cells (Chang *et al.*, 1992; Webb, 2000; Williams

and Holder, 2000). As hair cells differentiate, they arise in pairs having opposite polarities (180˚) with respect to the orientation of their kinocilia and stereocilia providing an axis of maximal sensitivity (Rouse and Pickles, 1991). Hair cells arising after the first primordial migration are oriented parallel with the anteroposterior axis of the fish body. The second primordial neuromast hair cells are oriented perpendicular to the anteroposterior axis of the fish body (Whitfield, 2005). The patterns of primary neuromast migration and deposition are highly consistent among larval teleost species, as compared between zebrafish, Mexican blind cavefish (*Astyanax fasciatus*), and medaka (*Oryzias latipes*) even though the adult neuromast distributions are remarkably dissimilar (Sapède *et al.,* 2002)**.**

Only superficial neuromasts are present in early and late-stage teleost larvae (Jones and Janssen, 1992). These neuromasts respond maximally to low frequencies in the range of 10-60 Hz (Montgomery *et al*., 2002) The specific range of detection is unknown for larval striped bass superficial neuromasts, however, they are able to detect *A. salina* nauplii which produce an antennal beat frequency of 8-10 Hz within 1-2 body lengths (Gallagher and Burdick, 1970; Barlow and Sleigh, 1980; MacIntosh & Duston, 2007). Mottled sculpin larvae (*Cottus bairdi*) feeding in the dark responded to prey even when buried in the substratum (Hoekstra and Janssen, 1985). When a section of the lateral line was coated with paste, responses to prey were reduced near the region of the covered lateral line. A limitation of this study was that prey capture efficiency was not quantified.

Presumptive canal neuromasts start off as superficial neuromasts which become enclosed within canals beginning in the late larval stage and is completed by metamorphosis to the juvenile stage (Webb and Shirey, 2003). Bony epithelial walls arise around presumptive neuromasts and fuse over individual neuromasts to form one segment of a canal (Fig. 1.4; Tarby and Webb, 2003). Segments grow toward each other leaving a pore between them (Webb, 1989a). Changes in water displacement at the pores are detected by the neuromast hair cells as the water enters the canal and stimulates movement of the hair cells in the direction of maximal sensitivity (Webb and Shirey, 2003). Adult piper (*Hyporhamphus ihi*) were most efficient at detecting and capturing prey swimming parallel to the lateral line than at other angles in the dark as observed

using infrared video (Montgomery, 1989). This behavioural evidence is congruent with anatomical findings that canal neuromast hair cells are oriented parallel to the lateral line (Münz, 1979). Neuromasts compensate for the lack of vision in the blind cavefish (*Astyanax hubbsi*), on which superficial neuromasts are twice as large and have longer cupula than the sighted river fish congener, *Astyanax mexicanus* (Teyke, 1990). However, chemoreception also compensates for the lack of vision as blind cavefish have three times as many taste buds at 22 dpf as sighted cavefish (Varatharasan *et al.*, 2009). The contribution of mechanoreception to non-visual feeding of larval striped bass is currently unknown and warrants investigation.

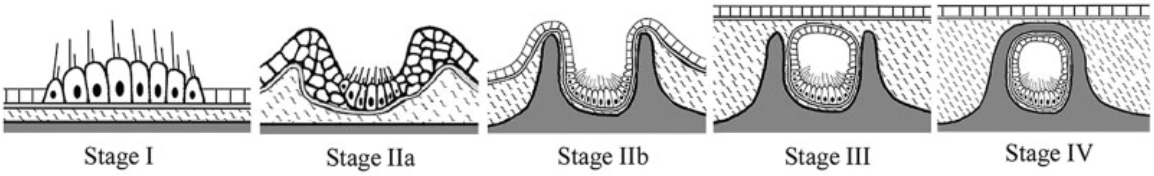

Figure 1.4 Schematic representation of canal segment development in the mandibular and supraorbital canals of the zebrafish. Stage I: Presumptive canal neuromast sitting on the epithelium above the thin underlying dermal bone. Stage IIa: Neuromast sits in a shallow depression. Stage IIb: The canal groove deepens as the ossified bony walls extend upward from the underlying bone, but remains open with the neuromast on the bottom. Stage III: Epithelium fuses over neuromast and encloses the canal. Stage IV: Ossified canal walls fuse over the neuromast to form a canal segment having an ossified canal roof. Image reproduced with permission (Webb and Shirey, 2003).

The function of neuromasts can be tested experimentally through ablation of neuromast hair cells using ototoxic chemicals (Coombs *et al.*, 2001; Montgomery *et al*., 2002; Engelmann *et al*., 2002). In adult mottled sculpin, the canal neuromasts are responsible for the orienting response to a vibrating object as this ability disappears with selective ablation of canal neuromast hair cells using gentamicin (Coombs *et al.*, 2001). In adult rainbow trout (*Onchorhynchus mykiss*), canal neuromasts were determined to mediate short-distance prey localization and capture in the dark as this ability was lost after ablation of the lateral line (Montgomery *et al*., 2002). Vibrations in the water column detected by the canal neuromasts allow adult mottled sculpin to orient themselves toward the vibration source (Coombs *et al.*, 2001). Both superficial and canal neuromasts are also capable of detecting the presence of low frequency vibrations or sound waves

produced from predators which is important for escape responses (Fuiman and Magurran, 1994).

The lateral line system is capable of adapting to specific hydrodynamic conditions of a habitat (Engelmann *et al.*, 2003). Both canal and superficial neuromasts of adult goldfish (*Carassius auratus*) and rainbow trout were able to detect signals from a vibrating sphere placed in still water (Engelmann *et al*., 2002). However, when low frequency background flow (noise) was increased, goldfish superficial neuromasts lost responsiveness to the signal of 50 Hz while the canal neuromast response was maintained (Engelmann *et al.*, 2002). Goldfish inhabiting still waters develop approximately 1000 superficial neuromasts on each body side and an additional 1000 on the head, which is more than the total number of canal neuromasts (Puzdrowski, 1989; Engelmann *et al.*, 2002). Trout, which inhabit running water, develop more canal neuromasts than superficial neuromasts (Engelmann *et al.*, 2002). Consequently, trout were able to maintain detection of the 50 Hz signal in running water better than goldfish (Engelmann *et al.*, 2002). Lateral line canal neuromasts provide sensitivity to signals in running water and are able to respond to a range of frequencies (40-200 Hz) depending on species (Montgomery *et al*., 2002; Bassett *et al.*, 2005). This finding also suggests that the canal neuromasts may be more efficient at detecting relatively high frequency vibrations and lose sensitivity to streaming water flows, while superficial neuromasts detect low frequency (10-60 Hz) vibrations indicative of smaller prey as well as water currents. Juvenile walleye pollock (*Theragra chalcogramma*) and sable fish (*Anoplopoma fimbria*) feeding in the dark were able to catch large *A. salina* prey (5 to 9 mm) more often than small prey (1.5 to 4 mm) possibly due to the larger water disturbances by the larger prey which increased detection (Ryer *et al.*, 2002). Recordings from lateral line sensory neuron stimulation of Antarctic fish neuromasts revealed that maximal sensitivity occurs at 40 Hz (Montgomery and MacDonald, 1987). In accordance with this finding, the limb beat frequency output by copepods, a major prey item of Antarctic fish, is 10-45 Hz, indicating that superficial neuromasts are suited for detecting movement of prey (Montgomery, 1989). The juvenile common bream (*Abramis brama*) feeds in total darkness on *Daphnia* with a reactive distance of 5 mm (Townsend and Risebrow, 1982). As canals form and mottled sculpin approach 29 dph, they become less sensitive to

vibrations from *A. salina* (Jones and Janssen, 1992). Striped bass larvae approaching the juvenile stage (17 to 25 dph) exhibited lower feeding incidence in the dark with increasing age (White, 2000). Canal neuromasts are better at detecting high frequency vibrations than the superficial neuromasts that dominate early larval stages and may not detect the low frequency output by *A. salina* metanaupli antennal beating (8-10 Hz) as easily (Jones and Janssen, 1992; Barlow and Sleigh, 1980). As fish grow, the shift toward detecting higher frequencies may complement the shift away from the pursuit of smaller prey items. These studies indicate that superficial neuromasts are capable of detecting the vibrations of swimming invertebrate prey at the larval stage and may be involved in non-visual feeding.

b) Audition

In seawater, the speed of sound depends on temperature, salinity, and depth (Mackenzie, 1981). At a temperature of 25˚C, salinity of 35 ppt, and depth of 1000 m, the speed of sound is about 1550 m s^{-1}, about five times the speed of sound in air. Sound has two major components: velocity (particle motion) and sound pressure (Hawkins, 1993; Lu *et al.*, 1996). Sound pressure travels through a medium of air and water in a series of rarefactions and compressions in which energy is passed from one particle to the next, causing the particles to bump into each other (particle motion). The particles also bounce back, regaining their original position (Myrberg and Fuiman, 2002). In air, the vibrations of nitrogen and oxygen molecules lead to the detection of sound pressure by the ear of terrestrial species which provides information about sound intensity, but not velocity (Higgs *et al.*, 2006). In water, sound pressure also provides information about intensity, but velocity (particle motion) provides information about intensity, as well as the direction of the sound (Myrberg and Fuiman, 2002). Particle motion consists of displacement, which is the distance each water particle moves, velocity, which is the speed of that movement, and acceleration, which is the rate of change in velocity (Myrberg, 2001).

Teleost audition involves an inner ear containing responsive hair cells and accessory organs, such as swimbladders (Fay and Popper, 1975; Popper and Lu, 2000). The sensory hair cells transduce acoustic information to electrical impulses (Popper and

Lu, 2000). The apical end of the hair cells contains rows of graded cilia from tallest to shortest which project into the lumen of the otolithic organ (Popper and Lu, 2000). Audition occurs when the shorter apical hairs, stereocilia, of the otolithic organ are bent towards the taller kinocilium resulting in depolarization, a similar mechanism to lateral line hair cells (Myrberg and Fuiman, 2002; Chang *et al.*, 1992). The otoliths possessed by teleosts are about three times denser than the body of the fish (Popper and Lu, 2000). In the presence of a sound, the recipient fish's flesh becomes displaced and oscillates at the same frequency as the vibration, while the denser otoliths lag behind, resulting in a deflection of the cilia (Hawkins, 1993).

Sound pressure indirectly stimulates the otolithic sensory hair cells by causing vibrations of the swimbladder, while direct stimulation occurs through particle motion generated by movement of the fish's body creating inertia of the otoliths (Kalmijn, 1989; Schwartz, 1985; Hawkins, 1993). The swimbladder is part of the acoustico-lateral line system and acts by amplifying the sound pressure component to the inner ear otolithic organ (Fay and Popper, 1975). Hearing capability based on sound pressure detection varies from species to species over a continuum (Popper and Fay, 2010). This continuum ranges from fish having a close connection between the swimbladder and otolithic organ (examples: squirrelfish, Holocentridae family; mormyrids, Mormyridae family; herring, Clupeidae family) to having no swimbladder at all and only relying on particle motion components of sound via the lateral line (examples: flatfish, Pleuronectidae family; gobies, Gobiidae family; elasmobranchs, Torpedinidae family). Fish having a close connection between their swimbladder and inner ear receive sound pressure signals from distant sources. Swimbladder inflation has been implicated in the initiation of the startle response and avoidance of predators as displayed in larval herring (*Clupea harengus*) suggesting full functionality of the larval inner ear to respond to sound information (Blaxter *et al.*, 1981). Striped bass larvae in particular fill their swimbladder between 5 and 7 dph (Doroshev and Cornacchia, 1979). It is difficult to define where different species of fish fall on the continuum of hearing specialization and has only been quantified for some species (Popper and Fay, 2010). Striped bass have been previously classified as hearing generalists due to the lack of specialization in the connection of the swimbladder to the otolithic organ, referred to as Weberian ossicles (Popper and Fay,

1993). However, it has recently been acknowledged that there is no evidence to suggest that fish lacking these specializations are hearing generalists (Popper and Fay, 2010).

It is currently unknown how well or how far striped bass adults can detect sound pressure, but they do give avoidance responses to frequencies below 1000 Hz even without Weberian ossicles (ESEERCO, 1991 cited in Popper and Carlson, 1998). A lower hearing threshold has been hard to determine experimentally, but the more turbulent the aquatic environment, the less sensitive fish are at detecting sounds and the auditory threshold is raised (Hawkins, 1993). A range of 50 to 1000 Hz is the predominant hearing range among most fish species, but infrasound (< 20 Hz) can also be detected by the ear (Enger *et al.*, 1993). Frequencies as low as 0.1 Hz have been detected by the ear in codfish (*Gadus morhua*) while detection by the lateral line was eliminated as the source was outside the short detection range of the neuromasts (Sand and Karlsen, 1986).

The prey of striped bass larvae in the wild are zooplankton, such as copepods (*E. affinis*) and cladocera (*Bosmina longirostris*; North and Houde, 2003), but cultured larvae are typically fed brine shrimp (*A. salina*) because of year round availability. Stage I *A. salina* fed to early larval stages have an average width, including appendages, of 497 μm and produce an antennal beat frequency of 8-10 Hz, which is too low to be detected by the fish ear (Barlow and Sleigh, 1980). Most research on the structure of fish hearing apparati fails to investigate the physiological or behavioural aspects which complement sound detection (Kingsford *et al.*, 2002). While hearing may provide striped bass larvae with important information about potential predators, hydrodynamic disturbances of small zooplankton are detected by the lateral line system having a reactive distance of 1-2 body lengths from the vibrating source, such as *A. salina* (Gallagher and Burdick, 1970; Karlsen and Sand, 1987; Hawkins, 1993; Popper and Lu, 2000). At a density of 100 prey l^{-1}, larvae would continuously be within 1-2 cm of a prey item assuming the food is randomly distributed (Gallagher and Burdick, 1970). Larval mottled sculpin superficial neuromasts responded maximally to frequencies below 30 Hz, while canal neuromasts responded maximally between 30 and 100 Hz indicating that low frequencies are most important for lateral line based prey detection (Coombs and Janssen, 1990). Overall, hearing is likely not useful for larval detection of small zooplankton prey as their

swimming appendages produce low amplitude vibrations below the frequency range of otolithic hair cells. However, neuromasts of the lateral line are well suited to detect these low frequency vibrations over a short distance.

c) Olfaction

Olfaction is a means by which fish sense migration routes, detect food, find mates, avoid predators, and recognize kin (Hara, 1994). The four main groups of compounds detected by olfaction in fish are amino acids, bile acids, gonadal steroids, and prostaglandins (Hara, 1994). The initial concentration of odorants released from a school of fish can influence the distance from which a predator can detect its prey. Turbulence may eliminate a weak odor from an individual, but may carry a more concentrated odor from a school of fish (Giske *et al.*, 1998). Through behavioural experimentation, it is often difficult to distinguish olfaction from gustation in fish because the sensory input from both is integrated within the telencephalon and diencephalon in such a way that complex behaviour is only observed when both taste and olfaction are intact (Kotrschal, 2000). Both olfactory and taste cells have been implicated in the detection of amino acids, but olfaction works longer distance. An example is of zebrafish responding behaviourally to amino acids diffused through water. When the nares were temporarily blocked, the behaviour was not observed even with functional taste cells (Braubach *et al.*, 2009). However, olfaction and taste also have certain functions that overlap. Specifically, olfaction is used in food search, discrimination of pheromones, and amino acids, and some alcohols, while taste is used to discriminate between amino acids and some alcohols (Kotrschal, 2000; Braubach *et al.*, 2009). However, sensory stimuli of similar nature can have different neural interpretations for either taste or smell depending on the biological function (Atema, 1980). For example, inhibiting the olfactory cells of bottom feeding ictalurid catfish prevented the detection of live prey, but not when the barbels were removed (Parker, 1910). This thesis will focus on the role of mechanoreception more than olfaction as it seems unlikely that young striped bass larvae would be capable of following a trail in turbulent or clay water, such as in the Shubenacadie estuary. Furthermore, the location of vibrations would allow spatial resolution not permitted by scent plumes. Adult fish may locate prey by following a scent

trail, but larvae likely wait in a concentrated food patch and strike upon detection of movement (Eldridge *et al.*, 1981). Therefore, it seems that mechanoreception may be relied on more than olfaction when visual information is not available as in dark or turbid conditions (Hara, 1994; Giske *et al.*, 1998). However, this hypothesis warrants testing as the non-visual feeding mechanism may vary between species with plaice relying on chemoreception to detect food more than mechanoreception (Batty and Hoyt, 1995).

d) Gustation

Gustation may also play a role in food detection, and some fish have taste cells located externally on the body in addition to inside the mouth (Hara, 1994). Taste is the final step in feeding and determines whether the food item will be accepted or rejected, but like all senses, is not as developed in larvae as it is in adults (Kasumyan and Doving, 2003). Many prey items and plants contain free amino acids which are capable of activating extra-oral gustatory cells and cause a response in the gustatory nerves of teleosts, as shown in an electrophysiological study (Dabrowski and Rusiecki, 1983; Marui and Caprio, 1992). Larval red sea bream (*Pagrus major*) use gustatory cells located around their open nostrils to locate patches of food before they are even capable of feeding (Tanaka *et al.*, 1991). They remain in the food patch until they are able to feed, presumably to save energy (Tanaka *et al.*, 1991). However, for some fish (carp, *Cyprinus carpio*; bitterling, *Rodeus sericeus*; black molly, *Poecilia sphenops*) most amino acids act as deterrents and for crucian carp (*Carassius carassius*) and Arctic flounder (*Liopsetta glacialis*), none are stimulatory (Kasumyan and Doving, 2003). The threshold concentration for a fish to detect a stimulatory substance by taste is 0.0001 to 0.01 M (Kasumyan and Doving, 2003).

Fathead minnow larvae are capable of feeding in the dark on live prey, but not as well on dead prey (Salgado and Hoyt, 1996). This finding suggests that while the use of olfaction and gustation may be involved for non-visual prey detection to some extent, vibrations may provide the most information about the location of food items, but again warrants investigation due to differences between species.

1.3 Ontogeny of the Lateral Line

The sensory systems of most vertebrates are derived from cranial placodes, which are areas of thick tissue on the head of developing organisms. Sensory neurons develop from their respective placodes and form into the lens of the eye and peripheral sensory receptors including the epibranchial gangla which innervate the face, olfactory neurons, otic neurons, and the lateral line neuromasts (Webb and Noden, 1993; Schlosser and Northcutt, 2001). In teleosts, the intricate details of primordial migration have only been described for zebrafish (Sapède *et al.*, 2002; Whitfield, 2005), while the ontogeny of neuromasts has been characterized for some teleosts including cardinal fish (*Apogon cyanosoma*), bullseye (*Parapriacanthus ransonetti*), northern anchovy, and sea bass (*Dicentrarchus labrax*; O'Connell, 1981; Rouse and Pickles, 1991; Faucher *et al.*, 2003). However, the ontogeny of striped bass neuromasts has not been characterized nor have the neuromasts been analyzed in terms of hair cell count or size. There is considerable variation of lateral line systems between species and eight different canal neuromast distribution patterns have been described within the teleost species alone (Webb, 1989b; Webb, 1990). The superficial neuromast distribution patterns are even more different between species, warranting the characterization of ontogeny for the striped bass lateral line system (Blaxter and Fuiman, 1989; Fuiman *et al.*, 2004). Axolotl (*Ambystoma mexicanum*) is a species of amphibian which serves as a useful model of developing placodes also observed in teleost larvae. Axolotl have five lateral line placodes which include the anterodorsal, anteroventral, middle, supratemporal, and posterior (Fig. 1.5 A and B; Schlosser and Northcutt, 2001).

Figure 1.5 A. Axoltl amphibian B. Lateral line placodes of the axolotl shown in black, other placodes are grey. Anterior is toward the left, dorsal toward the right. Abbreviations: ad, anterodorsal lateral line placode; av, anteroventral lateral line placode; fe, facial epibranchial placode; lp, lens placode; mid, middle lateral line placode; ol, olfactory placode; otv, otic vessel; pld, dorsal subdivision of posterior lateral line placode; plm, main subdivision of posterior lateral line placode; plv, ventral subdivision of posterior lateral line placode; st, supratemporal lateral line placode. Image reproduced with permission (Schlosser and Northcutt, 2001)[1].

On the cranium of larval fish, at least six lateral line placodes exist in two distinct locations referred to as pre-otic placodes (anterior to the otic placode), from which the cephalic neuromasts arise, and post-otic placodes (posterior to the otic placode), from which the trunk neuromasts arise (Webb and Noden, 1993). Lengthening of a placode is referred to as a migrating primordium. Several primordia migrate during the ontogeny of neuromasts in a succession of waves along a specific path. The first primordium migrates along the trunk in pre-hatch zebrafish depositing clusters of 7-9 neuromast precursor cells at intervals (Sapède *et al.*, 2002). Deposition of the first neuromast cells in zebrafish is complete by 2 days post fertilization (dpf; Fig. 1.6 A). Secondary neuromast precursor cells for intercalary neuromasts are deposited between the primary neuromasts, but develop at a slower rate (Grant *et al.*, 2005).

The trunk and tail of zebrafish, also a useful model for ontogeny of the teleost lateral line, are innervated by the posterior lateral line nerve while the dorsal part of the head is innervated by the dorsal anterior lateral line nerve, and the cheek and lower jaw are innervated by the ventral lateral line nerve (Bradbury *et al.*, 2008). As the migrating primordium leaves the post-otic placode, it is followed by the posterior lateral line ganglia which send out growth cones (Metcalfe, 1985; Whitfield, 2005). Glia derived

[1] Reprinted from the Journal of Comparative Neurology, 418, Schlosser, G. and Northcutt, R.G., Development of neurogenic placodes in *Xenopus laevis*. 121-146, copyright (2000), with permission from Elsevier.

from the neural crest also follow closely behind the ganglion axons (Gilmour *et al.*, 2002). The lateral line nerve, composed of the ganglia and glia, runs along the horizontal myoseptum (Whitfield, 2005). Glia inhibit the proliferation of neuromasts, therefore the lateral line neuromast cell clusters of the first primordial wave migrate away from the lateral line nerve by 3 dpf before they are able to proliferate and differentiate (Fig. 1.6 B; Ledent, 2002; Grant *et al.*, 2005). Also at 3 dpf, the second primordium migrates depositing neuromast and intercalary neuromast precursor cells between the first neuromasts (intercalation). These also move ventrally away from the lateral line to differentiate (Sapède *et al.*, 2002). By 5 dpf, the precursor intercalary neuromast cells from the first primordium have developed into neuromasts and second primordial neuromasts have differentiated (Fig. 1.6 C; Whitfield, 2005). In zebrafish, the primary neuromasts are deposited about five somites apart, the secondary neuromasts are deposited two somites apart, while the intercalary neuromasts are spaced about 1 to 2 somites apart (Ghysen and Dambly-Chaudiere, 2005). The significance of primordial migration and intercalation relates to the presence of neuromasts on striped bass larvae early in development. Presence of neuromasts at first feeding stages will indicate potential for use of mechanoreception during feeding. Intercalation as the method of lateral line development indicates that neuromasts being present at all points of the body and not concentrated in one area, such as the head, will increase the chance of prey detection by increasing the responsive range around the larvae.

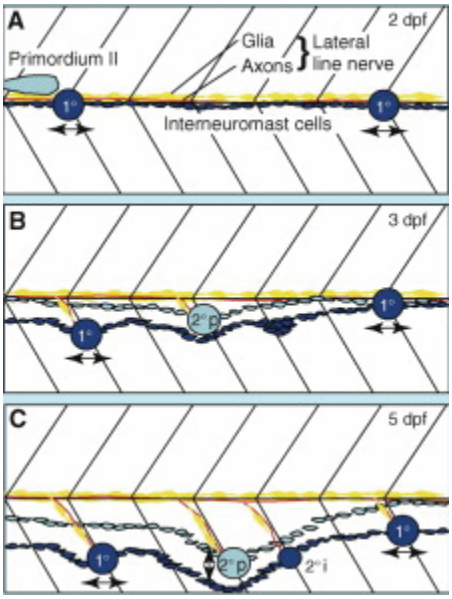

Figure 1.6 Model of neuromast deposition in larval zebrafish. At 2 dpf (A), the first primordial migration has occurred, deposition of the primary neuromasts (1˚) is complete, and the second primordial migration begins. At 3 dpf (B), the first neuromasts migrate ventrally away from the inhibitory glia (yellow) and the secondary primordium (2˚p) begins depositing precursor cells. At 5 dpf (C), the second primordial neuromasts are differentiated as well as the first primordial interneurons (2-i). Picture reproduced with permission (Whitfield, 2005)[2].

1.4 Neuromast Ablation and Staining

Ablation is one approach used to investigate the role of mechanoreception in non-visual feeding of fish. Research on neuromast ablation most commonly uses zebrafish as an animal model. Several of these studies are focused on ablating hair cells for the purposes of gaining information about hearing and balance in humans (Harris *et al*., 2003; Owens *et al*., 2007). Other studies investigate the structure of neuromast cells or their sensitivity to aminoglycoside antibiotics (Song *et al.*, 1995; Williams and Holder, 2000; Santos *et al.*, 2006). Aminoglycosides, including streptomycin, gentamicin, and neomycin, have been used in ablation studies due to their ototoxicity or ability to kill the hair cells of neuromasts and otolithic organ (Schacht, 1986; Kaus, 1987; Harris *et al.*,

[2] Reprinted from Current Biology, 15, Whitfield, T.T., Lateral line: Precocious phenotypes and planar polarity, R67-R70, Copyright (2005), with permission from Elsevier.

2003; Mukai, 2006). However, aminoglycoside exposure through an external medium leaves the neuromasts dysfunctional without affecting the inner ear otolithic hair cells (Karlsen and Sand, 1987; Murakami *et al.*, 2003). Only neomycin and gentamicin are known to activate the apoptosis pathway with certainty (Song *et al.*, 1995; Ylikoski *et al*., 2002; Van Trump *et al.*, 2010).

Aminoglycosides have both an internal and external mechanism of action; one is the binding of the aminoglycoside to negatively charged components of the cellular membrane, displacing calcium ions. However, this action is reversible and even antagonized by calcium or other divalent ions (Schacht, 1986). This point was of some concern in this thesis because striped bass larvae reared in brackish water require seawater mixed with the drug to survive exposure, but was dealt with by lowering the salinity from 5 ppt to 1 ppt. The second mechanism of action is irreversible and occurs after the aminoglycoside, such as neomycin, has entered the hair cell resulting in apoptosis (Schacht, 1986). Recent investigation of various aminoglycosides has indicated that there may be several different cell death pathways activated depending on the aminoglycoside used (Owens *et al.*, 2009). Six aminoglycosides (neomycin, gentamicin, streptomycin, kanamycin, tobramycin, and amikacin) have the same route of entry and the same initial rapid hair cell death, but some exhibited a slower process that continued to kill hair cells with longer exposure (Owens *et al.*, 2009).

Neomycin elicits a caspase-dependent pathway (rapid hair cell death) and a caspase-independent pathway (prolonged hair cell death; Matsui *et al.*, 2002; Owens *et al.*, 2009). In the caspase-independent pathway, neomycin increases permeability of mitochondrial membranes within each hair cell to Acyl coenzyme A binding protein (ACBP), allowing theinteraction of ACBP with the peripheral benzodiazepine receptor (PBR) to form a complex. The ACBP-PBR complex enhances the effect of calpain by reducing its calcium requirement (Fig. 1.7; Shulga and Pastorino, 2006). The ACBP-PBR complex and calpain also facilitate the release of apoptosis inducing factor (AIF) which enters the cell nucleus, interacts with several nucleases, such as Endonuclease G (EndoG), and forms an active DNA degradation complex (Takano *et al.*, 2005; Shulga and Pastorino, 2006; Hangen *et al.*, 2010). Calpain also feeds back positively on bid, a proapoptotic molecule, promoting the formation of truncated bid which is more potent in

perpetuating the release of calpain and AIF providing an apoptosis pathway independent of caspase activation. Calpain is involved in regulating both apoptosis and necrosis (Leist and Jaattela, 2001). Apoptosis is characterized by fragmented nuclei and darkened cytoplasmic contents indicative of neomycin damage resulting in caspase-independent pathway activation with acute exposure (15-90 minutes; Owens *et al.*, 2007; 2009). Necrotic-like characteristics can also occur resulting in apoptosis through caspase-dependent pathways with chronic exposure (3-6 hours) to aminoglycosides, such as neomycin (Owens *et al.*, 2007; 2009). Necrotic-like characteristics include swollen mitochondria and protrusion of apical hair cell ends (Owens *et al.*, 2007).

The caspase-dependent pathway involves bid acting on the mitochondria by forming pores in the membrane enabling the release of cytochrome c (Wei *et al.*, 2000). Cytochrome c then activates the formation of the caspase 9-Apaf-1 complex (Hengartner, 2000). This complex then activates caspase 3 which cleaves DNA into fragments resulting in apoptosis (Wang *et al.*, 2003; Sharma and Rohrer, 2004). Inhibiting Jun kinase or caspase 3, activating caspase 9, or the over expression of Bcl2 (anti-apoptotic factor) can slow down aminoglycoside-induced cell death (Matsui *et al.*, 2002; Wang *et al.*, 2003; Cunningham *et al.*, 2004).

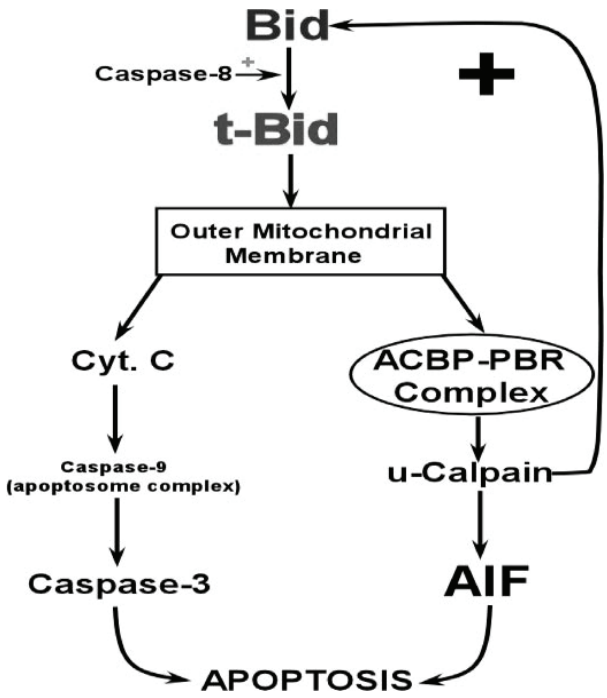

Figure 1.7 Model of apoptosis. Acyl coenzyme A binding protein (ACBP) and the peripheral benzodiazepine receptor (PBR) activate mitochondrial μ-calpain, which causes the release of the apoptosis inducing factor (AIF) from the mitochondrial intermembrane space which degrades DNA and also promotes the formation of truncated bid (t-bid). T-bid promotes release of cytomchrome c and activation of caspases perpetuating apoptosis. Cyt. C = cytochrome c. Image reproduced with permission (Shulga and Pastorino, 2006)[3].

Neomycin killed most neuromast hair cells in larval zebrafish within 30 to 90 minutes after which, no further decrease was observed. Gentamicin and kanamycin continued to kill hair cells in the second, slower process resulting in nearly all hair cells dying within three to six hours (Owens *et al.*, 2009). Three to six hours of drug exposure would not be feasible for work on striped bass larvae due to the need of conducting feeding trials afterwards and so neomycin was chosen over gentamicin. Whether neuromasts are the only sensory structure affected by aminoglycosides is one area lacking in knowledge. There are currently no reports on the effect of aminoglycosides on other sensory structures, such as taste buds or olfactory cells.

[3] This research was originally published in the Journal of Biological Chemistry. Shulga, N. and Pastorino, J.G. Acyl coenzyme A-binding protein augments bid-induced mitochondrial damage and cell death by activating μ-calpain. *Journal of Biological Chemistry.* 2006; 281: 30824-30833. © The American Society for Biochemistry and Molecular Biology.

To visualize neuromasts, many vital stains including FM1-43, FM1-43FX, DASPEI (2-(4-(dimethylamino)styryl)-N-ethylpyridinium iodide), DiAsp, and phalloidin, to name a few, have been used in many studies, particularly on zebrafish larvae (Harris *et al.*, 2003; Hernandez *et al.*, 2006; Owens *et al.*, 2008; 2009). FM1-43FX (N-(3-triethylammoniumpropyl)-4-(4-(dibutylamino) styryl pyridinium dibromide), a fixable dye used to label functional hair cells is used to show ablation of neuromasts (Gale *et al*., 2001; Ou *et al.*, 2007). FM1-43FX and aminoglycosides are taken up into hair cells via the same pathway, which is the mechanotransduction channel located at the tip of the kinocilia (Gale *et al*., 2001). Therefore, when hair cells are ablated via an aminoglycoside, such as neomycin, the mechanotransduction ability of the cell is lost and the entrance of FM1-43FX into the neuromast is inhibited (Gale *et al*., 2001). The result is a fainter glow or an absence of fluorescence under a fluorescent microscope. Olfactory cells are also targeted by FM-143FX and could be checked for fluorescence as well. For theses reasons, FM1-43FX was the stain chosen for experimentation on striped bass larvae.

A second staining method is immunohistochemistry in which a primary antibody is used to bind to specific proteins, such as calretinin, selectively found in taste cells and nerves, S100 protein, selectively found in neuromasts, and tubulin found in nerves (Grant *et al.*, 2005; Germanà *et al.*, 2007; Varatharasan *et al.*, 2009). Olfactory sensory cells co-express S100 and calretinin and so are visualized with primary antibodies targeting both S100 protein and calretinin (Germanà *et al.*, 2007). A secondary antibody, such as goat anti-rat, having a fluorophore attached, such as Alexa 488, is then added which binds to the primary antibody, as used for labeling zebrafish neuromasts by Harris *et al*. (2003). The fluorophore absorbs light at a specific wavelength and emits light at a longer wavelength enabling the visualization of the target cells (Mavrakis *et al.*, 2010). Many studies use several stains or staining methods to confirm visualization of neuromasts and their damage by aminoglycosides (Harris *et al.*, 2003; Hernandez *et al.*, 2006; Owens *et al.*, 2009).

Neuromast ablation data has mostly been confined to zebrafish larvae and focuses on effective concentrations and durations of exposure to neomycin. The highest neomycin concentration used to disrupt neuromast staining (DASPEI) was 0.5 mM

(Harris *et al.*, 2003). However, doses as low as 10 μM have been tested and found to cause damage to hair cells within zebrafish larval neuromasts (Williams and Holder, 2000). Durations of immersion in neomycin have ranged from 15 minutes to three hours (Harris *et al.*, 2003; Owens *et al.*, 2007). Neuromasts are damaged in a dose-dependent manner with only mitochondrial damage occurring with low doses (25 μM) and short exposure time (15 minutes), but more severe cellular damage and hair cell death occurring at higher doses (50 to 200 μM) and longer exposure times (30 to 60 minutes; Owens *et al.*, 2007). Maximal loss of staining occurred when a recovery (chase) interval was placed between neomycin exposure and staining (Owens *et al.*, 2007). However, 90 minutes after initial neomycin exposure in zebrafish larvae, hair cell death occurred regardless of concentration or duration of exposure (Owens *et al.*, 2009). These results were confirmed using both FM1-43FX and DASPEI stains.

In studies on non-visual feeding in fish, only four used ablation of both superficial and canal neuromasts (Jones and Janssen, 1992; Batty and Hoyt, 1995; Cobcroft and Pankhurst, 2003; Mukai, 2006). Of these four, only three studies performed scanning electron microscopy (SEM) to image neuromasts, olfactory cells, taste cells, the retina, and the inner ear otolithic organ, but none performed SEM or other histological procedures on larvae treated with streptomycin to determine if neuromasts and other sensory receptors were damaged (Jones and Janssen, 1992; Cobcroft and Pankhurst, 2003; Mukai, 2006). Therefore, existing research has not considered that olfaction and taste are potentially damaged by aminoglycoside treatment and reduced feeding in the dark may be due to the absence of these senses. Furthermore, these same four studies also used streptomycin despite the lack of knowledge surrounding appropriate concentrations and the extent to which hair cells were damaged. However, much data exists on neomycin in terms of effective concentrations, exposure times, and damage to hair cells using imaging tachniques (Williams and Holder, 2000; Harris *et al.*, 2003; Owens *et al.*, 2007; 2009).

In terms of behaviour, the effects of neomycin and streptomycin are similar (Kaus, 1987). Blinded adult striped panchax (*Aplocheilus lineatus*) were placed in either neomycin or streptomycin solutions ranging from 0.7 to 88 mg l^{-1} (< 0.1 mM) for 2 weeks and tested on alternate days (Kaus, 1987). For both drugs, no loss of ability to

locate a 50 Hz wave source was observed below 0.7 mg l^{-1}, but a significant reduction in this ability occurred above 5.5 mg l^{-1}, suggesting that either drug could be used in ablation and non-visual feeding trials as both have similar toxicity (Kaus, 1987). However, as more is known about the ototoxic effect of neomycin, it was chosen for this research study.

In predator avoidance studies, larval herring, plaice, cod (*Gadus morhua*), flounder (*Platichthys flesus*), and larval halibut (*Hippoglossus hippoglossus*) were treated with 10 mM streptomycin for either one to two minutes or tested directly in the streptomycin solution (Blaxter and Fuiman, 1989). A significant reduction in response to a probe in the dark was observed for all treatments of streptomycin using infra-red videography (Blaxter and Fuiman, 1989). Had the durations been longer, lower concentrations may have been found as effective. Behavioural tests of larval ability to respond to a probe after treatment could serve as a secondary means of determining effect of dose on mechanoreception. A behavioural test would also serve well to determine swimming ability and natural reflex after aminoglycoside treatment, but before a feeding trial.

Previous studies on prey detection using striped bass larvae 5 to 20 dph tested streptomycin concentrations of 1 to 2.5 mM in 3 ppt seawater for 4 to 10 minute intervals (MacIntosh, 2006). All doses above 1 mM affected swimming behaviour of the fish (MacIntosh, 2006). As no imaging of neuromasts was done after streptomycin treatment, it is unknown whether neuromasts were affected by the 1 mM streptomycin dose for 5 minutes or the extent of damage to the hair cells. Prey capture by 23 to 26 dph controls in darkness was 2.5 *A. salina* h^{-1} while for streptomycin treated larvae, prey capture was 1.8 *A. salina* h^{-1} indicating that neither group performed well or prey density was too low to obtain a significant effect. Prey capture rates were similar for larvae aged 9 to 10 dph feeding in the dark (MacIntosh, 2006). In the light, prey capture rates were comparable between treated and control larvae, indicating that streptomycin treated larvae retained the ability to feed, but evidence was not strong enough to suggest that mechanoreception was affected by streptomycin. Therefore, further research with striped bass larvae is required to determine appropriate concentrations and durations using neomycin to ablate neuromasts.

1.5 Overview of Rationale and Objectives

The ontogeny of neuromasts for striped bass larvae has not been characterized. By determining the specific ontogeny and timing of larval neuromasts in striped bass through to the juvenile stage (4 to 27 dph), the development and efficiency of larval feeding can be compared with the location and number of neuromasts present.

No studies on larval fish have determined appropriate aminoglycoside concentrations and exposure durations for effective neuromast ablation while conserving foraging behaviour. Neomycin was the aminoglycoside chosen for this thesis work due to the number of publications using neomycin in combination with imaging techniques which quantified neuromast damage (Williams and Holder, 2000; Harris *et al.*, 2003; Owens *et al.*, 2007; 2009). All visible FM1-43FX stained neuromasts were counted under a fluorescent microscope. To gain reliability for visualizing neuromasts, immunohistochemical methods were also performed along with confocal microscopy of several specimens to obtain detailed images of neuromast cellular damage. The use of two staining techniques, one a vital dye and one immunohistochemistry, provided greater certainty that neuromasts were dysfunctional when analyzed under a microscope and also provided confidence that the stains were targeting neuromasts.

To ensure that olfaction and taste were not affected by exposure to neomycin, imaging was done to determine whether olfactory bulbs and taste cells were still able to take up dye and therefore, fluoresce, suggesting functionality. The structure of neomycin treated taste cells was also imaged with the use of confocal microscopy to determine if damage occurred. This approach has not been reported in the primary literature in combination with neuromast ablation and feeding trials.

As a number of fish species exhibited behavioral changes to the presence of a probe in the dark following streptomycin treatment (Blaxter and Fuiman, 1989), larval striped bass behavior was monitored in this thesis study as a preliminary measure. Larvae treated with various neomycin doses were observed for changes in swimming responses to a series of taps on the side of a container. Larvae treated with neomycin of an appropriate dosage were then offered prey (*A. salina*) in both the light and dark at 10, 13, 17, and 20 dph. Larvae younger than 10 dph were not used due to their inability to

survive neomycin treatment. Larvae older than 20 dph were not used in feeding trials because of increased prey capture ability as the juvenile stage is approached. Initial experiments to determine effective neomycin concentrations without detrimental effects on visual feeding behaviour were required. If neuromasts have a role in prey capture, making them dysfunctional should reduce prey localization and capture. Ablation of larval striped bass neuromast hair cells provided an opportunity to quantify the difference in the number of prey caught which represents the magnitude that striped bass larvae depend on neuromasts for feeding in the dark as compared to in the light.

The use of 200 *A. salina* l^{-1} in this thesis is comparable to or less than prey densities found in the wild, making prey detection a challenge (Beaven and Mihursky, 1979; Setzler-Hamilton *et al.*, 1982). The stage I nauplii used in the prey capture experiment were of similar size to copepod species commonly found in the wild (Katona, 1971; Heinle, 1966).

Four studies using the aminoglycoside, streptomycin, in combination with feeding trials provide some evidence for the role of neuromasts in non-visual feeding (Jones and Janssen, 1992; Batty and Hoyt, 1995; Cobcroft and Pankhurst, 2003; Mukai, 2006). However, the lack of histology in these studies presents uncertainty as to whether the decrease in prey capture rates was due to dysfunctional neuromasts or the ablation of other sensory structures. Therefore, both fluorescent and confocal microscopy were performed to ensure ablation of neuromasts and to check for damaged olfactory and taste cells.

1.6 Objectives and Hypotheses Tested

Striped bass larvae were used in this thesis to investigate the following objectives:

1. Describe the ontogeny of striped bass larval neuromasts from 4 to 27 dph to compare with onset and progressive changes in feeding.

2. Effectively ablate the neuromasts by determining the appropriate neomycin concentration that will not inhibit visual feeding behaviour.

3. Establish efficacy of neuromast ablation and ensure maintainence of taste bud structural integrity through immunohistochemical staining and confocal microscopy.

4. Determine the effect of neuromast ablation on non-visual feeding at various stages of development (10, 13, 17 and 20 dph).

This research used striped bass larvae to test the following hypotheses:

H_0: Larvae treated with a neomycin dose that ablates neuromasts exhibit no inhibition of feeding, either in the light or dark.

H_A: Larvae treated with a neomycin dose that ablates neuromasts exhibit inhibition of feeding in the dark.

H_0: Neuromast staining with FM1-43FX dye is unaffected by exposure to neomycin doses ranging from 0.007 to 5 mM.

H_A: Neuromast staining with FM1-43FX dye is inhibited by exposure to neomycin in a dose dependent manner.

Chapter 2 General Methods

2.1 Egg Collection and Hatching Conditions

The larvae were hatched from eggs collected in May and June of 2009 and 2010 from the Stewiacke River (1 km upstream from the Shubenacadie/Stewiacke River confluence). Eggs were collected in a 1000 μm mesh plankton net over a 4 week period to provide cohorts of larvae. Eggs were held in Coleman coolers (7.6 l) supplied with upwelling aeration from a small aquarium pump (Elite) and transported to the NSAC.

Green, 80 l conical tanks were used to incubate the eggs at a temperature of 16-17˚C and salinity of 1-3 ppt. A water inflow rate of 200 ml min^{-1} provided an upwelling current to keep the eggs suspended while an air stone placed in the bottom provided additional lift. Salinity was controlled by mixing a supply of seawater (30 ppt) with fresh well water. Eggs hatched about 48 hours after collection. Larvae were transferred at 3 dph to one of six dark green, cylindrical tanks (120 l) in a small re-circulation system. Rearing temperature was held around 20˚C and salinity was 5 ppt. Temperature, salinity, and oxygen were regularly recorded between 08:00 h and 22:00 h. Oxygen levels averaged 100% saturation (range 95 to 110%). The photoperiod was L:D 12:12 with lights coming on at 08:00 h. Lighting was supplied by 3 shaded incandescent bulbs approximately 2 m above the tanks which provided dim light (about 50 lx) at the water surface.

First feeding commenced between 5 and 7 dph. Stage I *A. salina* nauplii were fed from age 5 to 15 dph. Stage II nauplii enriched with Algamac (Biomarine Aquafauna, California) were fed from 16 to 23 dph. Settled waste was siphoned at least once daily and oil was removed from the surface using Styrofoam skimmers to promote swimbladder inflation.

2.2 Artemia Salina Culture Methods

A. salina was the only food fed during the rearing of the larvae. In feeding experiments, only *A. salina* stage I nauplii were fed. Starting at 13:00 h each day, 60 ml of cysts were incubated in 12 l Plexiglas cones filled with sea water and kept at 28˚C

within a well-lit fiberglass cabinet. A fan heater was used to maintain the temperature in the cabinet and a silica diffuser supplied aeration to each cone. Hatching of stage I nauplii occurred within 16-20 h. The mean (SE) length of stage I nauplii (n=10) was 460 ± 50 μm and width, including appendages, was 497 ± 78 μm. The nauplii were harvested by placing a black plastic bag over the cone for complete darkness, removing the air stone, and placing a flashlight slightly above the base of the cone. As *A. salina* nauplii are positively photo tactic, they were attracted to the flashlight. After waiting approximately 20 minutes to allow unhatched cysts to settle to the bottom, the nauplii were siphoned into a bucket, then poured through a Nitex mesh filter (60 μm) and rinsed with freshwater to remove *Vibrio* bacteria. Nauplii were then transferred to 2 l containers of sea water, each containing an air stone, ready to be fed to larvae. Unused nauplii were stored in 4˚C water to prevent them from maturing and fed later that day. Other nauplii were incubated and enriched to reach stage II.

Enrichment of stage I nauplii was performed in aerated 1.8 l cones each having approximately 150, 000 nauplii. Cones were filled with 750 ml sea water heated to 28˚C to which 150 ml of *A. salina* were added at a concentration of approximately 1000 nauplii ml^{-1}. Algamac, 0.12 g, was emulsified for 2 minutes in 100 ml of sea water using a hand blender (Braun). The final volume of the enriched cones was about 1 l *A. salina* nauplii which were incubated for 12 h, then harvested at stage II. The *A. salina* were poured into a filter, rinsed, and diluted in sea water for immediate use or stored in chilled water as previously described. The mean (SE) length of stage II nauplii was 670 μm ± 215 μm and the width, including appendages, was 640 μm ± 66 μm.

2.3 Neomycin Sulfate Solutions

For initial ablation trials in 2009, a stock solution of 1.4 mM neomycin sulfate (908.9 MW) was made by dissolving 0.127 g neomycin sulfate with dH_2O to reach an end volume of 100 ml. Appropriate dilutions were made to concentrations of 0.007, 0.03, 0.1, and 0.7 mM with 5 ppt seawater (SW). For final trials in 2010, a stock solution of 10 mM neomycin was made by weighing 0.909 g neomycin sulfate and making to a final volume of 100 ml. Appropriate dilutions were made in concentrations of 1, 2, and 5 mM

in 1 ppt seawater. Brackish water diluent was made by mixing seawater trucked in from Sandy Cove (Institute of Marine Biosciences) and filtered with a 30 μm sandfilter with fresh well water to the desired salinity (1 or 5 ppt) measured by YSI handheld meter (Model #85). Neomycin solutions were made with brackish water because striped bass larvae have an absolute requirement for salinity. Rearing larvae in freshwater resulted in high mortality (Lal *et al.*, 1977)

Chapter 3 Ontogeny of Striped Bass Neuromasts

3.1 Introduction

Despite being primarily visual feeders, striped bass larvae inhabiting in the turbid Shubenacadie River estuary are likely relying on other senses to detect prey due to reduced visual acuity during the day due to high turbidity and complete darkness at night (MacIntosh and Duston, 2007). There is evidence that mechanoreception aids in non-visual feeding for larval mottled sculpin (*Cottus bairdi*), juvenile sole (*Solea solea*) and plaice (*Pleuronectes platessa*), larval striped trumpeter (*Latris lineata*), and larval willow shiner (*Gnathopogon elongatus caerulescens*; Jones and Janssen, 1992; Batty and Hoyt, 1995; Cobcroft and Pankhurst, 2003; Mukai, 2006). The ontogeny of neuromasts in larval striped bass (*Morone saxatilis*) has never been described. An important aspect of biological and physiological research is to relate structure to function. As a necessary prelude to ablation studies, it is also essential to map the location of the neuromasts as was done for larval willow shiner (Mukai and Kobayashi, 1995). At the time of hatching, the latter had approximately 21 cephalic neuromasts and 17 trunk neuromasts (Mukai and Kobayashi, 1995).

How neuromasts arise has been described in detail for a few species, such as zebrafish and axolotl, and provides a basis for naming striped bass neuromasts (Raible and Kruse, 2000; Schlosser and Northcutt, 2001; Harris *et al.*, 2003). In pre-hatched fish and amphibians a number of cranial placodes give rise to the posterior and anterior lateral line nerves and neuromast precursor cells (Cod *Gadus macrocephalus*, Otsuka and Nagai, 1997; African clawed frog *Xenopus laevis*, Schlosser and Northcutt, 2000; Axolotl *Ambystoma mexicanum*, Schlosser and Northcutt, 2001; zebrafish *Danio rerio*,Whitfield, 2005). In zebrafish, the anterodorsal placode gives rise to supraorbital (above eye), infraorbital (below eye), and otic (ear) neuromasts while the anteroventral placode gives rise to the mandibular (jaw) and opercular (along the opercular fin) neuromasts (Fig. 3.1; Raible and Kruse, 2000). The two post-otic placodes, middle and posterior, give rise to the middle lateral line neuromasts on the head and the occipital dorsal (upper head) and posterior trunk neuromasts, respectively (Raible and Kruse, 2000). The supraorbital region includes the preoptic and supraorbital neuromasts. The infraorbital region includes

the mandibular, infraorbital and opercular neuromasts. The caudal–cranial region includes the otic, occipital, dorsal and middle neuromasts. The posterior neuromasts are located in the trunk region (Fig. 3.1, Harris *et al.*, 2003). Although the ontogeny of placodes is similar between anamniotes, the time frame of deposition and maturation of neuromasts differ between species. In this chapter, striped bass neuromast ontogeny was drawn, neuromasts were named according to anatomical location, and specific structure of neuromasts was described.

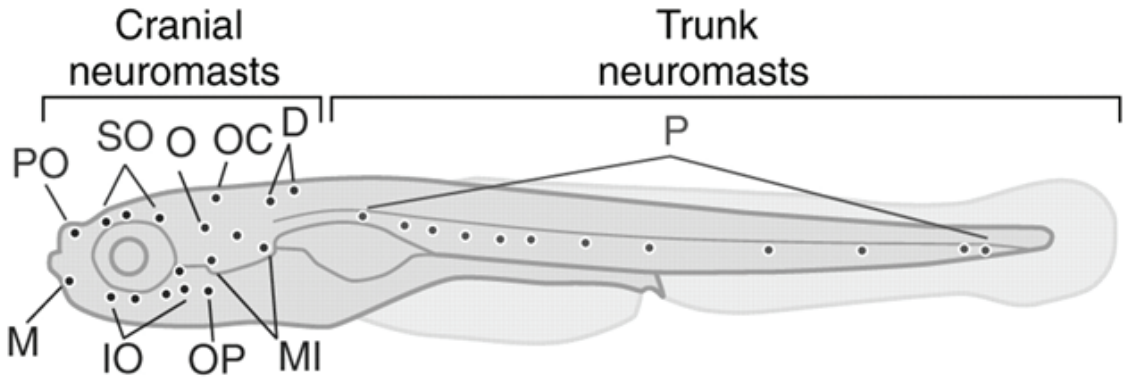

Figure 3.1 Schematic of neuromast distribution based on 4 to 5 dpf zebrafish larva from the lateral view. The supraorbital region includes the preoptic (PO) and supraorbital (SO) neuromasts. The infraorbital region includes the mandibular (M), infraorbital (IO) and opercular (OP) neuromasts. The caudal–cranial region includes the otic (O), occipital (OC), dorsal (D) and middle (MI) neuromasts. The posterior (P) neuromasts are located in the trunk region. Image reproduced with permission (Harris *et al.*, 2003)[4].

Hatching of zebrafish occurs at 3 days post fertilization (dpf) followed by first feeding around 4 dpf (Brown, 1997; Rowe *et al.*, 1998). In zebrafish, 4 dpf is the equivalent of 1 dph for striped bass. At the time of hatching, zebrafish larvae have about twelve trunk neuromasts and total of eight infraorbital and supraorbital neuromasts (Harris *et al.*, 2003). With such a well developed lateral line at the time of hatching and the need to feed within 24 hours, there is potential for the use of mechanoreception at first feeding of zebrafish and possibly for striped bass larvae as well. Since larval fish

[4] Journal of the Association for Research in Otolaryngology, 4, 2003, 219-234, Neomycin-induced hair cell death and rapid regeneration in the lateral line of zebrafish (*Danio rerio*), Harris, J., Cheng, A.G., Cunningham, L.L., MacDonald, G., Raible, D.W., and Rubel, E.W., Figure 1B, with kind permission of Springer Science and Business Media.

undergo large changes in morphology, physiology, and behaviour it was essential to describe the changes in striped bass neuromast number, size, and location and relate these to feeding ability.

3.2 Experimental Methods

The larvae used in 2009 were from three cohorts age 4, 7, 10, 13, 15, 17, 20, 23, and 27 days post hatch (dph) and ranging from 7 to 17 mm TL. To stain neuromasts, one vial containing 100 μg of FM1-43FX, a light sensitive vital dye (Invitrogen; Burlington, Ontario), was mixed in 2 ml double distilled water (dd H_2O). Another 52 ml of dd H_2O was then added and stirred with a glass stir stick to make a final FM1-43FX volume of 54 ml and concentration of 3 μM. Cell culture strainers (Falcon, 100 μm mesh, height 1.9 cm, 2.5 cm diameter) were fashioned into baskets by melting a hole in the extended side lip with a heated needle and gluing a sewing pin in place for a handle. This handle allowed the baskets containing larvae to be moved quickly between solutions to minimize stress. One well of a six well tissue culture plate (17 ml total volume each) was partially filled with 6 ml of FM1-43FX. A basket containing four to five larvae (N=4-5) was placed in FM1-43FX for 40 minutes in a dimly lit room (~200 lx) then transferred to tricaine methanesulfonate (TMS or MS-222) to euthanize larvae. For 10, 13, 17, and 20 dph, three baskets of four larvae each were examined (N=12). MS-222 was made by adding 0.6 g MS-222 to 50 ml fresh water and making to a volume of 2 l. Seawater was then added to a final volume of 3 l and final salinity was 10 ppt. Larvae were then immersed in paraformaldehyde fixative (PFA) at 4 ºC overnight and kept in darkness. This process was repeated for each larval age. PFA was made to a concentration of 4% by adding one ampule (10 ml) of 16% PFA (Cedarlane, Ontario) to a beaker and diluting with 20 ml of 0.2 M (Phosphate Buffered Saline- PBS) and 10 ml of dH_2O. PBS was made by adding 0.2 M of dibasic solution to 0.2 M of monobasic solution to a pH of 7.4 and stored at room temperature. Dibasic solution required the addition of 85.2g of sodium hydrogen phosphate (Na_2HPO_4, Sigma-Aldrich) and 48 g of sodium chloride (NaCl, Sigma-Aldrich) to 2 l dH_2O made to a final volume of 3 l. Monobasic solution required the addition of 27.6 g of sodium dihydrogen phosphate (NaH_2PO_4, Sigma-Aldrich) and 16 g of NaCl to 500 ml dH_2O made to a final volume of 1 l. After fixing, whole larvae

were stored in 2 ml amber centrifuge tubes light-proofed by tinfoil and refrigerated at 4 ˚C in PBS diluted to a concentration of 0.1 M with dH_2O. Neuromasts were observed the following day using a Leica MZ FLIII fluorescent microscope equipped with a coolSNAP-Pro Digital Camera. One flank of each larva was viewed in the lateral plane only and the number of neuromasts counted in the following divisions: eye (infra- and supra-orbital), preoptic (nose), mandibular (jaw), lower head (pre-opercular and opercular neuromasts), upper head (occipital and middle neuromasts), posterior (trunk), and terminal (tail). Supratemporal neuromasts were apparent, but occurred more dorsally on the head and were not included in the count. Total larval length was measured at time of imaging. The pattern of neuromast ontogeny was described and diagrams were hand-drawn using average neuromast counts as a starting point and comparing at least four larvae from digital images. The drawings were scanned and traced in Paint Shop Pro x2 (Version 12.50).

To establish neuromast structure and to compare neuromast size and hair cell counts, confocal microscopy was performed on select larval ages due to limited samples (11, n=3 neuromasts; 13, n=1 neuromast; 20 dph, n=2 neuromasts). Whole larval fixed samples of various ages were transferred from PBS to a solution of 2% dimethyl sulfoxide (DMSO, Sigma Aldrich), 1% bovine serum albumin (BSA), 1% normal goat serum (NGS), and 0.25% Triton X-100 (TX) in PBS (PBS-T) and left overnight at 4 ˚C in preparation for immunohistochemistry. Larvae were then incubated with a primary antibody diluted in PBS-T. Anti-S100 (rabbit; Glostrup, Denmark) synaptic marker specific for olfactory cells and neuromasts was used diluted 1:150 and anti-tubulin (monoclonal DM1A mouse antibody; Sigma) specific for nerves and kinocilia was used diluted 1:150 and was simultaneously labeled with S100 (rabbit; Abbate *et al.*, 2002). However, magnification was not high enough to see the kinocilia clearly. Anti-calretinin (mouse; Bellinzona, Switzerland) a marker for calcium binding proteins was also used to stain the lateral line nerve diluted 1:150. Larvae remained in primary antibodies for one week, at which time the samples were given four, 60 minute washes in PBS then placed in the secondary antibody solution. Goat anti-rabbit containing a fluorophore sensitive to light in the blue light range of 488 nm was used diluted 1:200 and goat anti-mouse containing a fluorophore sensitive to light in the green light range of 555 nm was used

diluted 1:200. Samples were incubated for 96 hours, given three 60 minute washes in PBS, then stored in PBS overnight and viewed the next day, initially with a Leica DM4000 B microscope equipped with epifluorescence. Blue (470/40 nm, visualization of 488 nm fluorophores as green) and green (546/10 nm, visualization of 555 nm fluorophores as red) excitation filters were used. (See page 26 for justification of neuromast, taste, and nerve visualization using immunohistochemical staining). Selected preparations of neuromasts from 11, 13, and 20 dph larvae were then viewed and photographed using a Zeiss LSM 510 META laser scanning confocal microscope. Negative controls were conducted in which larvae were incubated in secondary antibodies without first being labeled with primary antibodies. This resulted in little to no fluorescence in any sensory structures. Stacks of 10–20 images made by sequences of focal planes at 1–2 μm intervals were overlaid to create the images using projections created by the Zeiss LSM 510 software. Images were adjusted for brightness using Corel Paint Shop Pro x2 (Version 12.50).

3.3 Results

3.3.1 Ontogeny of Neuromasts

The distribution of larval neuromasts had similar patterns when stained with FM1-43FX and S100 antibody (Fig. 3.2, 3.3). The neuromasts were composed of tight cell clusters distributed on the head, trunk, and tail with a neuromast positioned on the point of the somites and intercalary neuromasts located in between (Fig. 3.2 A and B). All somites (about 40) gained their associated neuromasts by the juvenile stage (27 dph) and many neuromasts were located in between.

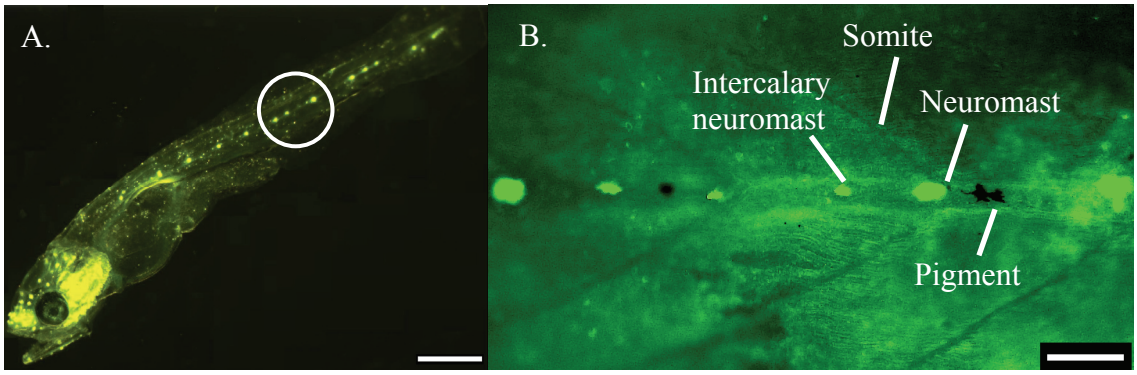

Figure 3.2 A. FM1-43FX stained 10 dph striped bass larva showing the neuromast distribution (green dots) Scale bar is 1 mm White circle indicates area shown in B. B. S100 stained 11 dph larva showing trunk neuromasts resting on the somite points. Smaller intercalary neuromasts are visible. Black spots are pigment. Scale bar is 100 µm.

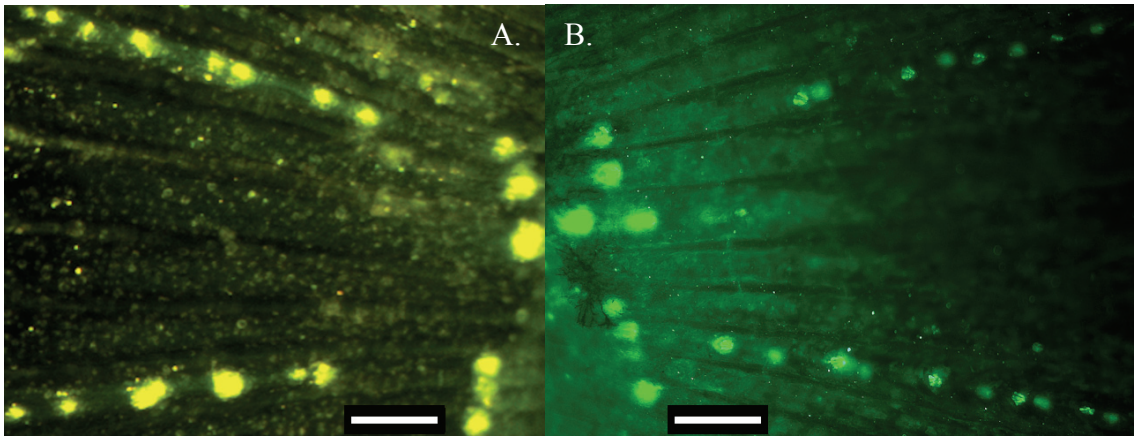

Figure 3.3 A. FM1-43FX stained 20 dph striped bass larva tail showing neuromasts (light green dots). B. S100 stained 20 dph larva tail showing the same neuromast distribution (dark green dots). Both scale bars are 100 µm.

The lateral line nerve, stained with calretinin, extended onto the tail (Fig. 3.4 A and B) where projections of afferent fibres were observed to indicate the position of neuromasts (Fig. 3.4 C). The lateral line nerve at 14 dph (Fig. 3.5 A) followed the same distribution pattern of neuromasts on the tail of 15 (Fig. 3.5 B) and 20 dph larvae (Fig. 3.5 C).

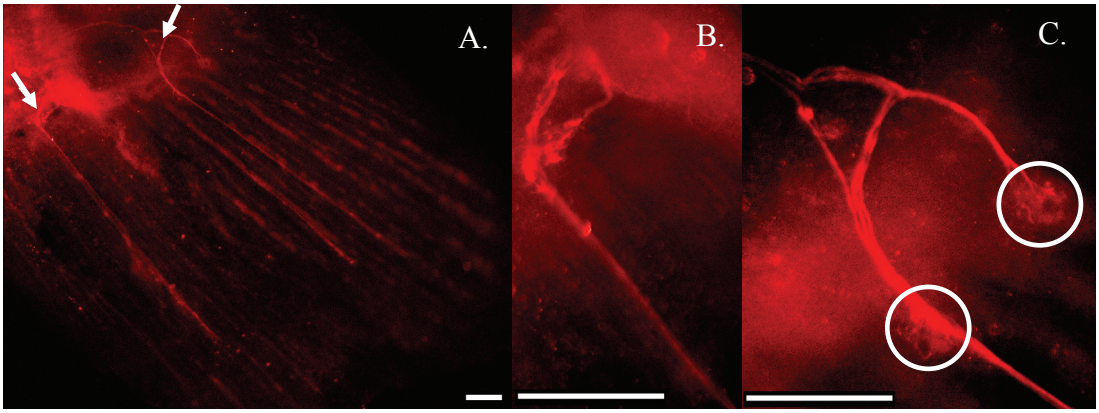

Figure 3.4 A. Lateral line nerves (arrows) stained with calretinin projecting onto the tail of a 14 dph striped bass larva treated with 5 mM neomycin. B. and C. show higher magnification of the tail lateral line nerves and projections of afferent fibres (white circles) indicating positions of neuromasts. All scale bars are 100 µm.

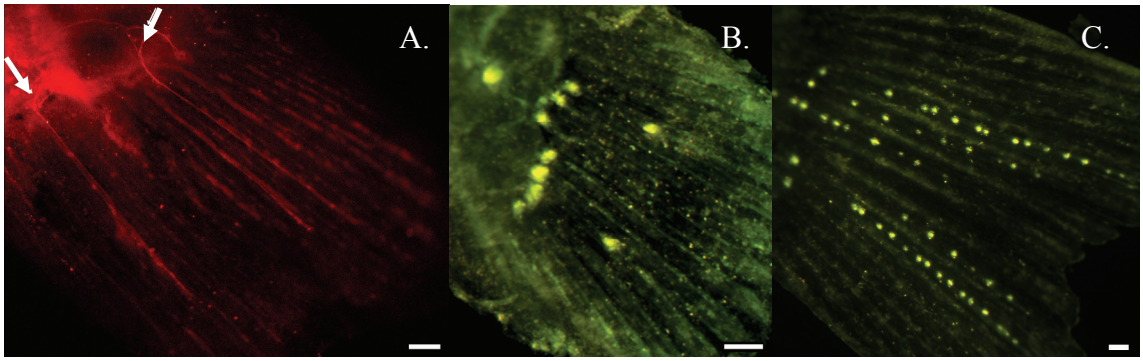

Figure 3.5 A. The lateral line nerve (arrows) of a 14 dph striped bass larva treated with 5 mM neomycin and stained with calretinin follows the lines of neuromasts found on the tail. B. Neuromasts on the tail of a 15 dph control larva stained with FM1-43FX. C. Neuromasts on the tail of a 23 dph control larva stained with FM1-43FX. All scale bars are 100 µm.

At age 4 dph (5.3 mm mean TL), the first supraorbital and infraorbital neuromasts were present anteriorly, as well as the first occipital neuromast while eight neuromasts appeared regularly spaced along the trunk (Fig. 3.6). By 7 dph (6.5 mm mean TL), the first terminal neuromast was present on the tail and approximately 5 (± 1 SE) more neuromasts had developed on the trunk. At 10 dph (7.5 mm mean TL), the number of anterior neuromasts had increased to approximately 13 (± 0.6 SE) including the addition of three pre-opercular, three pre-optic, and four mandibular, while two additional terminal neuromasts appeared on the tail. Trunk neuromasts were situated at the point of the somites, which were now visible. At 13 dph (9.6 mm mean TL), the number of

anterior neuromasts increased to 18 (± 3 SE), specifically in preoptic, mandibular, and preopercular neuromasts. At 15 dph (9.8 mm mean TL), all 13 infraorbital and supraorbital neuromasts were present anteriorly and more were present on the tail (Fig. 3.7). At 17 dph (11.4 mm mean TL), about three opercular neuromasts were present. At 20 dph (14.0 mm mean TL), the distribution was approaching that of the adult and many more neuromasts were added along the trunk, occurring at the point of each somite as well as in between each somite. By 27 dph (18 mm mean TL), the fish were beginning metamorphosis to the juvenile stage having an average of 63.5 (± 3 SE) trunk neuromasts, 45 (± 2 SE) cephalic neuromasts, and 43.5 (± 2 SE) tail neuromasts (Table 3.1). The tail was last to develop its full array of neuromasts (Fig. 3.7). Neuromast distributions depicted in Fig. 3.6 and 3.7 are more representative of typical rather than average distributions for the purpose of demonstrating where the neuromasts occur at various ages. Therefore, the number of neuromasts on the diagrams in Fig. 3.6 and 3.7 represent all observed neuromast locations and may not match the average numbers presented, although averages were taken into consideration.

The cephalic neuromasts of striped bass larvae followed the infraorbital, supraorbital, middle, and preoperculomandibular presumptive canal lateral lines. Neuromasts following the supratemporal lateral line were apparent, but appeared dorsally and most were not visible from the lateral view. As only the lateral view was used for this research and the supratemporal lateral line was relatively small, the supratemporal lateral line neuromasts were not counted. The trunk neuromasts followed a single lateral line posteriorly.

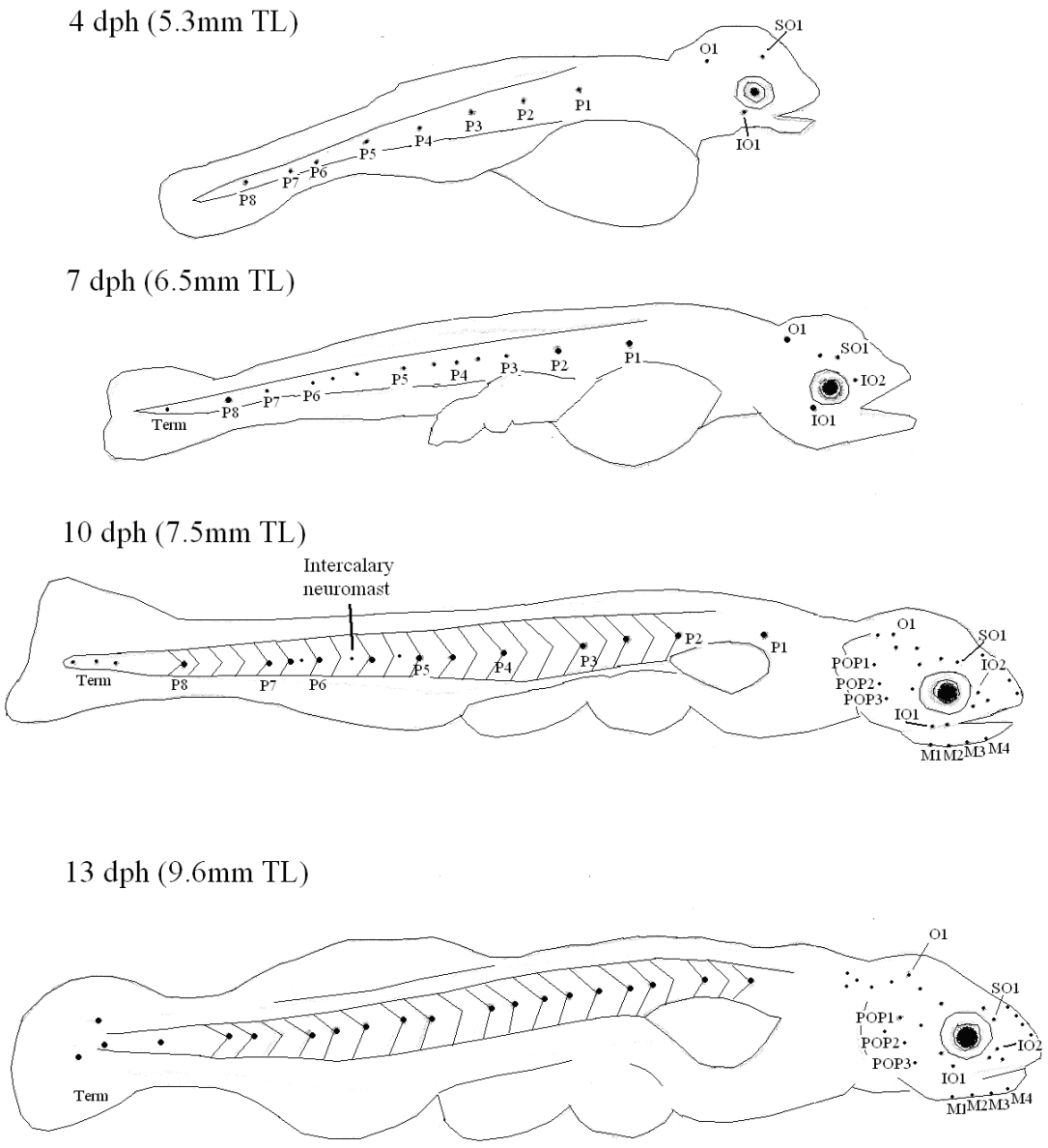

Figure 3.6 Ontogeny of striped bass larval neuromasts from 4 to 13 days post hatch (dph). 4dph: early neuromasts present. 7 dph: one terminal and five additional trunk neuromasts present. 10 dph: three preopercular, four mandibular, three preoptic neuromasts present and somites differentiated. 13 dph: five additional anterior neuromasts present on average. Early neuromasts are labeled IO1-2, infraorbital neuromasts; M1-3, mandibular neuromasts; O1, occipital neuromast; OP1-3, opercular neuromasts; POP1-4, pre-opercular neuromasts; SO1, supraorbital neuromast; P1-8, posterior trunk neuromasts; Term, terminal neuromasts of the tail. Intercalary neuromasts were occasionally apparent between neuromasts shown for 10 dph, but were not included in the counts as they were rarely observed and were much smaller than mature neuromasts.

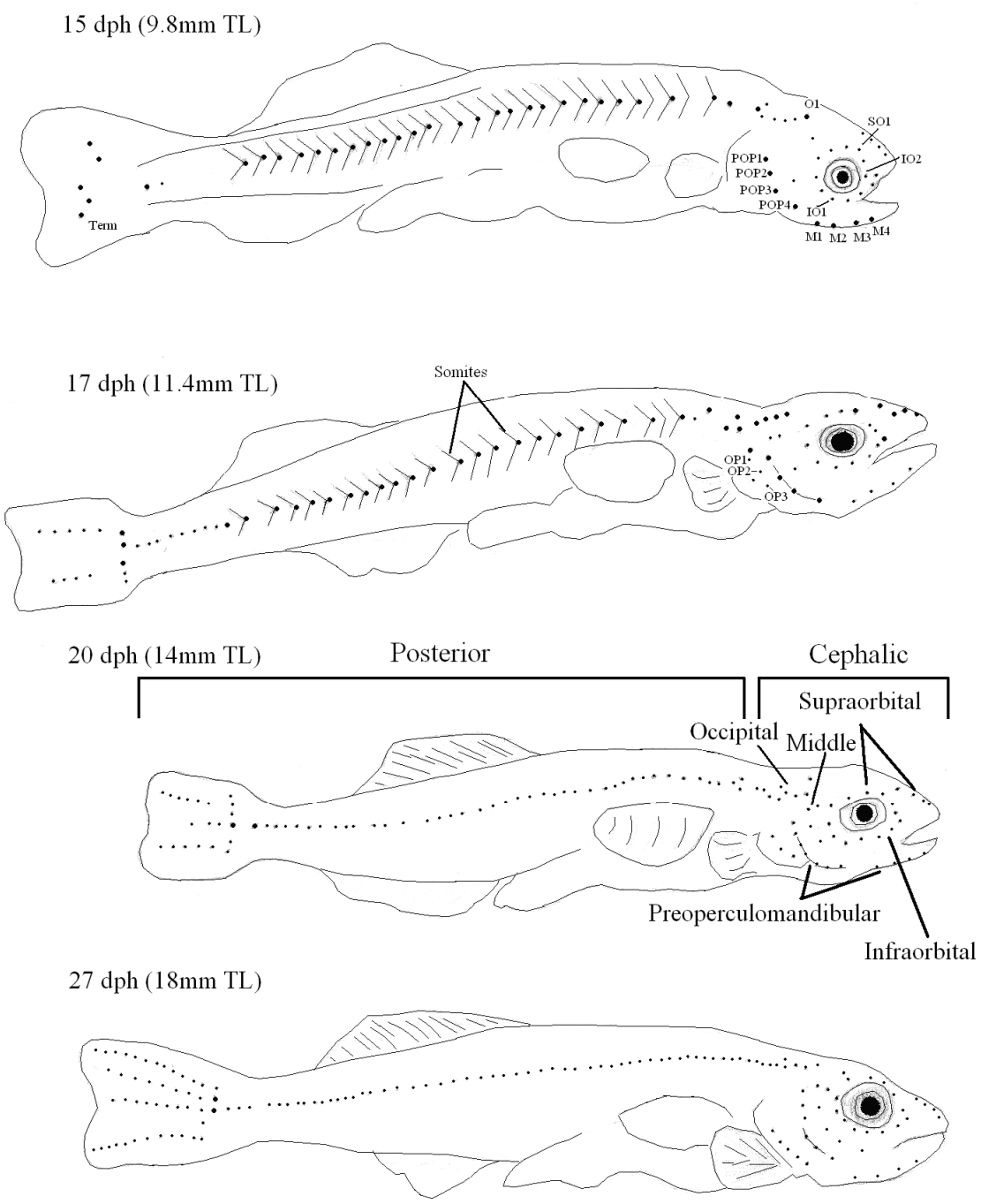

Figure 3.7 Ontogeny of striped bass larval neuromasts from 15 to 27 days post hatch (dph). 15 dph: all 13 infraorbital and supraorbital neuromasts present. 17 dph: three opercular neuromasts present. 20 dph: shows the infraorbital, supraorbital, middle, and preoperculomandibular lateral lines on the cephalic region and the posterior lateral line. 27 dph: approaching the juvenile stage showing the full array of neuromasts. Early neuromasts are labeled IO1-2, infraorbital neuromasts; M1-3, mandibular neuromasts; O1, occipital neuromast; OP1-3, opercular neuromasts; POP1-4, pre-opercular neuromasts; SO1, supraorbital neuromast; P1-8, posterior trunk neuromasts; Term, terminal neuromasts of the tail.

Table 3.1 Average anterior, trunk, and tail neuromast counts (± SE) and mean total lengths (range) for 4 to 27 dph striped bass larvae. N=total number of larvae observed.

Age (dph)	N	Anterior (± SE)	Trunk (± SE)	Tail (± SE)	Mean TL (Range)
4	4	3.75 (± 0.75)	7.5 (± 0.57)	1.25 (± 0.25)	5.3 (4-6)
7	5	3.4 (± 0.97)	8.75 (± 1.15)	2.6 (± 0.28)	6.5 (6-7)
10	12	13.2 (± 0.60)	11.4 (± 0.07)	3.3 (± 0.18)	7.5 (6.5-8.1)
13	12	18.1 (± 3.2)	22.4 (± 1.10)	2.85 (± 1.00)	9.6 (8-11.2)
15	4	27.75 (± 1.24)	20.7 (± 2.84)	4.5 (± 0.29)	9.8 (9-11)
17	12	21.75 (± 1.96)	35.75 (± 2.47)	12.8 (± 1.08)	11.4 (10.3-12.3)
20	12	26.4 (± 1.29)	44.3 (± 0.70)	18.1 (± 2.62)	14.0 (13.3-15.1)
23	4	45 (± 2.16)	59.75 (± 3.82)	36.25 (± 3.43)	16.6 (16-17)
27	4	45 (± 2.16)	63.5 (± 3.28)	43.5 (± 1.76)	18.0 (17-19)

3.3.2 Neuromast Structure

S100 stain and confocal microscopy allowed for detailed descriptions of neuromast composition. All cephalic neuromast images were taken from similar positions posterior to the eye. Cephalic neuromasts (n=3) from a 11 dph larva stained with S100 had an average hair cell count of 30, length of 46 μm, width of 26 μm, and projecting 12 μm out from the body (Fig. 3.8 A and B; Appendix Table A.1). A diagram shows the structure of a typical neuromast composed of hair cells, hairs, and nerve axons in Fig. 3.8 C. Neuromasts from 20 dph larvae were not as clearly defined, possibly due to thicker tissue and poor uptake of stain. Therefore, hair cells could not be counted. Cephalic neuromasts from a 20 dph larva (n=2) were about twice as long as on the 11 dph larva, being 90 μm in length, 57 μm in width, and projecting about 32 μm out from the body (Fig. 3.9 A and B; Table 3.1).

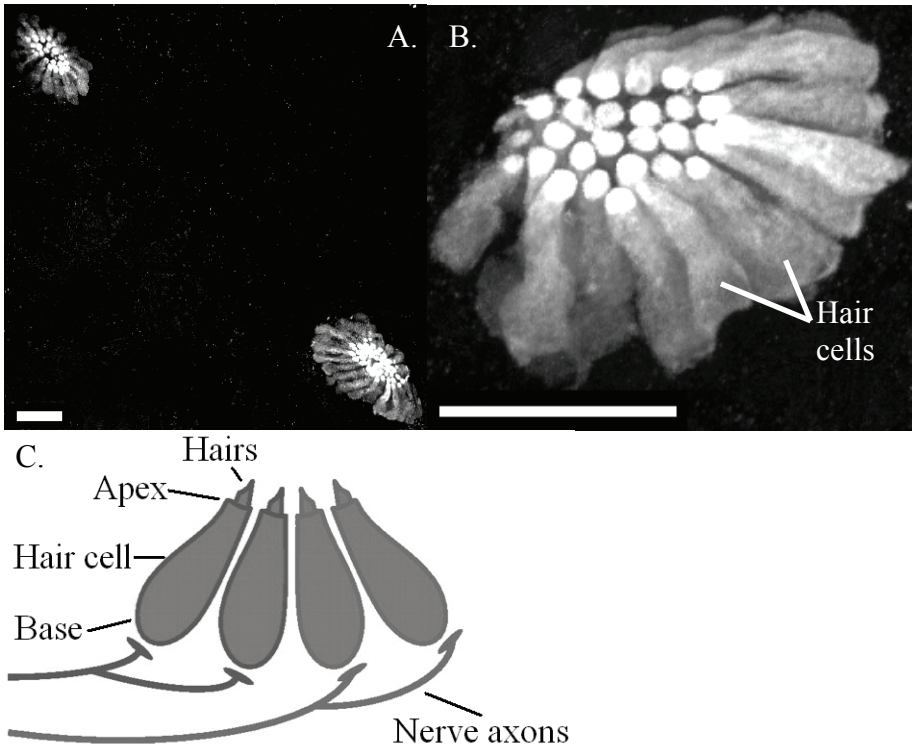

Figure 3.8 A. Confocal images of two cephalic neuromasts from a 11 dph striped bass larva 143 µm apart and stained with S100. Top left: length 43 µm, width 26 µm, height 14 µm, hair cell count 28. Bottom right: length 57 µm, width 29 µm, height 8 µm, hair cell count 32. B. A third cephalic neuromast of the same 11 dph larva stained with S100 with a hair cell count of 30. Length 39 µm, width 24 µm, height 15 µm. Both scale bars are 20 µm. C. Diagram of the lateral view of a neuromast showing hair cells. Hairs and nerve axons are not visible in confocal images.

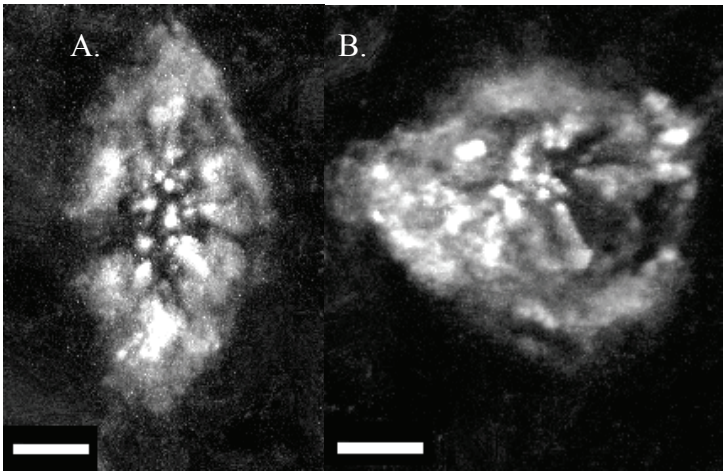

Figure 3.9 A. Cephalic neuromast from a 20 dph larva stained with S100. Length 93 µm, width 57 µm, height 30 µm. B. A second cephalic neuromast from the same larva. Length 86 µm, width 56 µm, height 33 µm. Both scale bars are 5 µm.

A 13 dph larval trunk neuromast had 12 hair cells, compared to 27 found in the cephalic neuromasts of the same larva (Fig. 3.10 A and B). However, despite the difference in hair cell number, cephalic and trunk neuromasts were similar in size at the larval age of 13 dph (Appendix Table A.1). A cephalic neuromast from a 13 dph larva measured 34 μm in length, 25 μm in width, and 21 μm in height and had 27 hair cells (Table 3.2). The trunk neuromast of the same larva measured 41 μm in length, 22 μm in width, and 22 μm in height, but had only 12 hair cells. Notches at the apical tip correspond with the position of kinocilia and show that the hair cells are oriented 180˚ to each other.

Table 3.2 Average cephalic neuromast size (μm) and hair cell counts for 11 (n=3), 13 (n=1) and 20 (n=2) dph striped bass larvae.

Age (dph)	Length (μm)	Width (μm)	Height (μm)
11	46	26	12
13	34	25	21
20	90	57	32

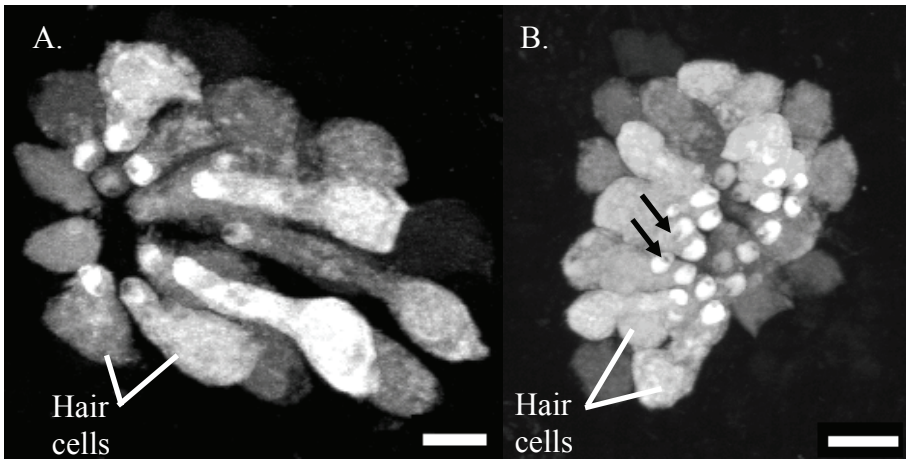

Figure 3.10 A. Trunk neuromast of a 13 dph striped bass larva stained with S100 with 12 hair cells. Length 41 μm, width 22 μm, height 22 μm. B. Cephalic neuromast stained with S100 located behind the eye of the same larva having 27 hair cells (length: 34 μm, width: 25 μm, height: 21 μm). Kinocilia appear as dark notches (black arrows) in the brightly stained S100 cuticular plate and are oriented at 180˚ to each other. Scale bars are 5 μm.

The lateral line nerve innervates the neuromasts (Fig. 3.11 A). Axons from the lateral line nerve bundle protruded up into the hair cells of the trunk neuromasts (Fig. 3.11 B).

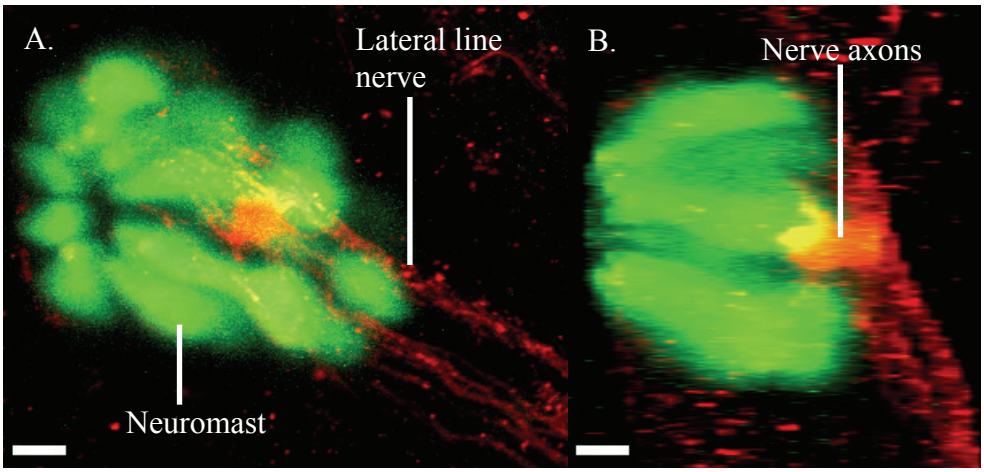

Figure 3.11 A. Trunk neuromast of a 13 dph striped bass larva having 12 cells and stained with S100 (green) sitting on top of its innervating lateral line nerve stained with tubulin (red). B. Same trunk neuromast rotated to show the nerve bundle protruding into the hair cells. Scale bars are 5 μm.

3.4 Discussion

This study provided the first documentation of larval striped bass neuromasts including those present at first feeding through to the juvenile stage and new knowledge as to the pattern of neuromast distribution. These results are part of a larger study designed to test the role of neuromasts in non-visual feeding. Neuromasts increased in number to 8 cephalic and 9 trunk neuromasts by the time of first feeding around 5 to 7 dph indicating they may serve a function, such as facilitating non-visual feeding. By comparison, European flounder (*Platichthys flesus*) had no cephalic neuromasts at 6 -7 dph, while Atlantic herring (*Clupea harengus*) had 6 cephalic neuromasts, and 15 trunk neuromasts at 6-7 dph, which is twice as many as striped bass at 7 dph (Blaxter and Fuiman, 1989). These differences are likely due to different rates of development of the fish, but also differences in sensory adaptations in specific environments. Larval Pacific herring (*Clupea harengus pallasi*) inhabiting estuaries where turbidity can reach 100 mg l^{-1} were capable of feeding in laboratories in turbid water up to 1000 mg volcanic ash l^{-1}

whereas Atlantic herring spawn in either shallow or deep tidal waters also turbid in nature (Boehlert and Morgan, 1985; Reid *et al.*, 1999). The turbid habitats of both Atlantic and Pacific herring larvae are similar to that of striped bass larvae, which suggests that the abundance of neuromasts early in development could be useful for non-visual feeding. At the time of hatching for willow shiner inhabiting calm lakes, there were many neuromasts present (21 cephalic and 17 trunk neuromasts), but this species does not begin to feed until 7 dph and is blind early on (Mukai and Kobsysshi, 1995). Therefore, mechanoreception likely contributes to predator detection and avoidance for this species from 1 to 7 dph. At 7 dph, mechanoreception likely contributes to their ability to feed in complete darkness (Mukai and Kobayashi, 1995; Mukai, 2006). On the other hand, weakfish larvae (*Cynoscion regalis*) which inhabit clear water do not develop superficial neuromasts until 13 dph, but begin to feed at 8 dph (Connaughton *et al.*, 1994). Neuromasts only develop along the trunk and not on the head of these larvae, suggesting that neuromasts may not be as important when light is available and visual feeding is possible (Connaughton *et al.*, 1994). At the other end of the spectrum, neuromasts and chemoreception are utilized for non-visual feeding in the blind cavefish, on which superficial neuromasts and taste buds are abundant, indicating a contribution of mechanoreception to feeding in darkness (Teyke, 1990). By the juvenile stage (27 dph), the number of striped bass neuromasts had increased to 30 cephalic, 60 trunk, and 45 tail neuromasts. As striped bass inhabit the turbid Shubenacadie estuary where little visual information is available, these larvae could also potentially benefit from a well-developed lateral line system for non-visual feeding early on.

Striped bass larval cephalic neuromasts were larger in general than trunk neuromasts for any given age and also had more hair cells, with an average of 30 hair cells at 11 dph. In blind cavefish, the superficial neuromasts are larger and have longer cupulae than those in sighted fish, which presumably improves the sensitivity of neuromasts (Teyke, 1990). Likewise, larval striped bass cephalic neuromasts having more hair cells than trunk neuromasts may indicate the importance of cephalic neuromasts in detecting swimming invertebrate prey. However, cupular lengths of striped bass larval neuromasts should be measured to determine a potential correlation with neuromast size.

On the trunk, chevron-shaped myotomes (somites) containing fast, white muscle fibers were clearly observed by 10 dph, but were also likely present much earlier, facilitating the escape response (Sänger and Stoiber, 2001). Slow, red fibers differentiate as a thin layer on top of the white fiber layer, while the intermediate fibers develop by first feeding in zebrafish and red seabream (*Pagrus major*), but not until the juvenile stage for sea bass (*Dicentrarchus labrax L.*; van Raamsdonk *et al.*, 1982; Matsuoka and Iwai, 1984; Scapolo *et al.*, 1988; Sänger and Stoiber, 2001). By 27 dph, neuromasts were located on each somite point with intercalary neuromasts located between points. By contrast, in other species such as zebrafish, blue tilapia (*Oreochromis aureus*), and convict cichlids (*Cichlasoma nigrofasciatum*) the location of intercalary neuromasts are strictly located on the somite points (Webb, 1989a; Ghysen and Dambly-Chaudiere, 2007).

As striped bass larvae aged from 13 to 20 dph, cephalic presumptive canal neuromasts became enlarged and elongated which is typical of canal neuromast formation (Webb and Shirey, 2003). Among larvae older than 20 dph, cephalic neuromast images were not clear enough to obtain hair cell counts. Therefore, it is unknown whether cephalic neuromasts remained larger than trunk neuromasts at this larval stage, but could be determined with other imaging methods, such as scanning electron microscopy. Each canalicular neuromast develops an axis of maximal sensitivity parallel to the direction of the canal (Webb and Shirey, 2003). The formation of canals has been postulated to prevent sensory fatigue of the neuromasts which may occur from continuous water flow over body surfaces (Dijkgraaf, 1962).

The notches appearing in the brightly stained S100 neuromast apex were comparable to kinocilia roots from other studies using confocal microscopy on zebrafish larval neuromasts, both stained and unstained (Lopez-Schier *et al*., 2004; Nagiel *et al.*, 2009). The orientation of striped bass kinocilia roots at 180˚ to each other was similar to zebrafish (Rouse and Pickles, 1991; Lopez-Schier *et al*., 2004; Nagiel *et al.*, 2009). Kinocilia of juvenile sea bass (*Dicentrarchus labrax*) hair cell bundles of both superficial and canal neuromasts were observed oriented at 180˚ to each other along the neuromast axis, but only in some cases (Faucher *et al.*, 2003). Other hair bundles had random orientations relative to the neuromast axis (Faucher *et al.*, 2003). An unexplored detail of

striped bass larval neuromasts is how the axis of hair cell and neuromast orientation changes with somatic location. Adult piper (*Hyporhamphus ihi*) canal neuromast hair cells are often oriented parallel to the lateral line as supported by infrared video in which capture was more efficient when prey were traveling parallel to the lateral line than at other angles (Münz, 1979; Montgomery, 1989). The direction of maximal sensitivity on the head and trunk of striped bass larvae is still unknown, but could provide useful information as to how they detect prey.

Chapter 4 Effect of Neomycin on Neuromast Ablation

4.1 Introduction

Aminoglycosides which target neuromast hair cells have been widely used to study neuromast and hair cell function, as well as neuromast contribution to feeding behaviour. The effects of streptomycin on feeding have been tested on only a few fish species in both the light and dark (willow shiner larvae (*Gnathopogon elongatus caerulescens*); Mukai, 2006; striped trumpeter larvae (*Latris lineate*); Cobcroft and Pankhurst, 2003; juvenile sole (*Solea solea*) and plaice (*Pleuronectes platessa*); Batty and Hoyt, 1995). Only one study used imaging of larval sculpin (*Cottus bairdi*) superficial and canal neuromasts in combination with feeding trials (Jones and Janssen, 1992). However, neuromasts were not imaged following streptomycin treatment and so confirmation of neuromast damage was not obtained. In preliminary work at NSAC, larval striped bass (*Morone saxatilis*) age 5 to 20 dph were exposed to relatively high doses of streptomycin (1 to 2.5 mM) for short periods of exposure (4 to 10 minutes) in an attempt to inhibit neuromast function (MacIntosh, 2006). However, these doses most often resulted in death of the larvae indicating the importance of finding a suitable dose before conducting feeding experiments. The results from these experiments have been inconsistent in terms of response variables as prey capture was not always quantified. The lack of imaging methods to confirm neuromast ablation in these studies has kept the effect of streptomycin ambiguous.

In contrast, neomycin has been studied extensively in other fishes and is known to have specific effects on neuromasts. Neomycin doses used on larval zebrafish (*Danio rerio*) ranged from 10 μM to 0.5 mM (Williams and Holder, 2000; Harris *et al.*, 2003), while durations of immersion in neomycin have ranged from 15 minutes to three hours (Harris *et al.*, 2003; Owens *et al.*, 2007). Neuromasts are damaged in a dose-dependent manner with only mitochondrial damage occurring with low doses (25 μM) and short exposure time (15 minutes; Owens *et al.*, 2007). More severe cellular damage and hair cell death occur at higher doses (50 to 200 μM) and longer exposure times (30 to 60 minutes; Owens *et al.*, 2007). Neuromasts of 10 dpf zebrafish larvae after a 3 hour immersion in 10 μM of neomycin exhibited central hair cell death and a compensated

increase in the turnover rate of peripheral cells (Williams and Holder, 2000). Beginning 4 h after a one hour exposure to neomycin, all neuromasts exhibited reduced staining of the hair cells. Uptake of stain did not return to control levels until 24 hours later. However, following low neomycin doses (<0.5 mM), hair cell uptake of DASPEI was greater at 4 h and continued to increase at 12 h and 24 h after exposure suggesting the steady recovery of hair cells (Harris *et al.*, 2003). Neuromast ablation in 5 dpf zebrafish larvae was most effective when a recovery interval (chase) of 1 hour was placed between aminoglycoside exposure and staining (Owens *et al*., 2007). A recovery interval of 3 hours was used by Harris *et al.* (2003), while a recovery interval of 2 hours was used by Ma *et al.* (2008). Despite the extensive literature on the effects of neomycin on neuromasts, its effects on feeding ability of fish have never been reported. Therefore, the objective was to determine an effective neomycin dose and duration for striped bass larvae to evaluate the impact of non-functional neuromasts on prey capture.

To determine an effective dose for ablating neuromasts a variety of histological methods have been used. Neuromast ablation is often quantified by scoring individual neuromasts or hair cells or counting total visible neuromasts. DASPEI, a stain which targets mitochondria, was used on 4 to 5 dpf zebrafish larvae treated with varying concentrations of neomycin for 60 minutes. Complete elimination of staining was observed at the highest concentration of 0.5 mM (Harris *et al.*, 2003). Stained neuromasts were then scored on a visibility scale as absent (0), reduced staining (1) or present (2) using a template typical of the larval age investigated (4-5 dpf; Harris *et al.*, 2003). However, the template was not constructed by Harris, but was taken from a previous study and did not account for individual variation among the fish tested. It was common practice for neuromast or hair cell counts to be checked with a second stain, such as phalloidin, to ensure reliability of initial counts (Harris *et al.*, 2003). A second method previously implemented was the counting of visible neuromasts simultaneously positive for two different stains (FM1-43 and DiAsp; 4-(4-(diethylamino)styryl)-*N*-methylpyridinium iodide), thereby improving reliability (Hernandez *et al.*, 2006). A third method implemented was to stain neuromasts using FM1-43FX and represent the counts of neomycin treated neuromast hair cells at four locations only (SO1, supraorbital 1; SO2, supraorbital 2; OC1, occipital 1; O1, otic 1) as percentages of the control neuromast

hair cell counts (Owens *et al.*, 2008). An alternative approach was to score 10 specific neuromasts on each larva as with DASPEI staining and fluorescent microscopy (Owens *et al.*, 2009). The objective for this thesis was to use two staining methods to provide reliability that neuromast damage had occurred with neomycin treatment; one a vital dye method using FM1-43FX, taken up by hair cells and one an immunohistochemical method using calretinin, S100 protein, and tubulin (Grant *et al.*, 2005; Germanà *et al.*, 2007; Ou *et al.*, 2007). (See page 25 for justification of neuromast, taste, olfactory sensory cell, and nerve visualization using immunohistochemical staining). Neuromasts were scored as bright or faded, but to reduce subjectivity in this thesis, the data was presented in two ways; the first with brightness scores only and a more conservative method with the total number of visible neuromasts. An effective dose was defined as that which would not impair larval swimming ability and also reduced staining of neuromasts under a microscope, either in terms of brightness or the total number visible.

Neomycin treatment may affect swimming ability. A decrease in escape response to a probe in the dark was observed for several species of larvae treated with streptomycin (>0.7 mg l^{-1}) using infra-red videography, but prey capture ability was not recorded for this experiment (Blaxter and Fuiman, 1989). Observation of swimming ability was used in this study to test whether dose of neomycin affected behaviour. Examination of other sensory modalities (taste buds and olfactory pits) for intactness after aminoglycoside treatment also warranted investigation as none of the aforementioned studies tested this possibility.

4.2 Methods

4.2.1 Neuromast Ablation and Staining of Neuromasts, Olfactory Cells, and Taste Buds

An appropriate neomycin dose and duration to effectively ablate the neuromasts was determined. Larvae were gently captured from their rearing tanks with flour sifters (15 cm diameter), placed in a white bucket of approximately 3 l rearing water, and transported to the experimental room where the lights were off, but the door was kept

open to maintain enough visibility to work (~200 lx). A flashlight was used to see while counting larvae into cell strainers using a 120 ml plastic cup. Transparent larvae were visible only by their black eyes. Cell strainers were sitting in a plastic square container (13.5 x 13.5 x 3.5 cm) filled with just enough rearing water (20 ºC) that the larvae could not jump out. Larvae were randomly distributed between twelve strainers (four concentrations x three replicates per age) by gently pouring one or two larvae from the cup into a cell strainer and moving on to the next until each contained four larvae. Excess water was removed with a disposable pipette so strainers would not overflow. Strainers were used to facilitate movement of larvae between solutions. Six welled BD Falcon tissue culture plates (Fisher Scientific, Part # 353224) were used in 2009 trials. Three wells of four plates contained 6 ml of neomycin sulphate solution, 5 ppt SW bath, FM1-43FX, or MS-222. Seawater containing calcium had the potential to prevent neomycin from effectively killing hair cells (Schacht, 1986), but it was necessary to make neomycin solutions with seawater to keep the striped bass larvae alive. Therefore, the concentration of seawater used to make neomycin solutions was lowered from 5 ppt in 2009 to 1 ppt in 2010.

In 2009, three strainers were placed in 6 ml neomycin sulfate baths (0.007, 0.03, 0.1, or 0.7 mM; Sigma, product # 1405-10-3) made with 5 ppt seawater (SW) or in a control bath of 5 ppt SW. Durations of 30 and 60 minutes were tested on four larval ages (10, 13, 17, and 20 dph) and the recovery interval was 90 minutes.

Determining the appropriate neomycin dose was resumed in 2010. This time 1, 2, and 5 mM doses made with 1 ppt SW were tested using 1 ppt as a control, and the duration of neomycin exposure was set at 60 minutes. A recovery interval of two hours was placed between neomycin treatment and staining.

Three white plastic containers (height: 5 cm, diameter: 12.5 cm) were filled with 150 ml neomycin solution (1, 2, or 5 mM) and one was filled with a 1 ppt SW control (Fig. 4.1). The higher volume used in 2010 compared to 2009 was an attempt to maximize exposure of the larvae to the drug as more solution surrounded the cell strainers, but the volume within each strainer was the same as in 2009. The seawater controls and baths for each treatment were set up in the same way. To conserve the amount of FM1-43FX used, the smaller tissue culture plates were employed instead of

the large containers for this step, as well as for the MS-222 euthanasia. Blue, translucent recycling bags were placed over the container tops to minimize disturbance during the trial, preventing stray light from the flashlight or door from inactivating the FM1-43 dye and neomycin treatments.

Three strainers, each containing four larvae, were placed in each neomycin bath (1, 2, or 5 mM) or a control bath of 1 ppt seawater for 60 minutes (Fig. 4.1). After 60 minutes, strainers and larvae were lifted from the treatment solutions, given two rinses in 1 ppt SW baths for 20 seconds each, then placed in 1 ppt SW for two hours to allow sufficient time for neomycin treated neuromast hair cells to die. Strainers were then transferred to FM1-43 FX for 40 minutes and covered with blue plastic containers to keep stray light out. The trial ended by transferring the strainers to MS-222 to euthanize the larvae (Fig. 4.1). Larvae were then stored in PFA and examined within one week. Total lengths were measured and swimbladder presence was noted. To determine the effect of neomycin dose, all visible neuromasts on the head and trunk of one lateral side for each larva were counted under a fluorescent microscope and scored as either bright or faded.

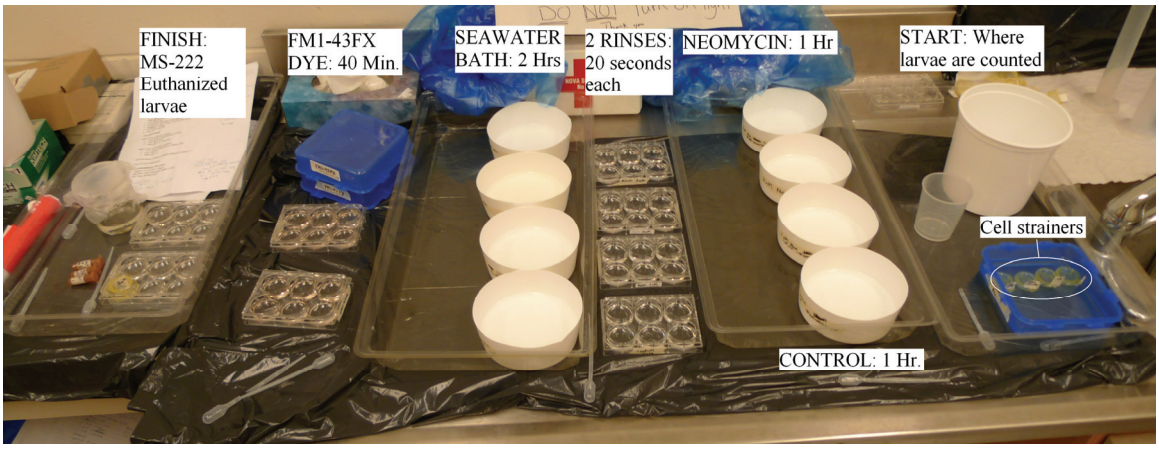

Figure 4.1 Experimental set-up for ablation of striped bass neuromasts conducted with the lights off and larvae transferred between solutions in cell strainer baskets (Falcon) in the right to left direction.

4.2.2 FM1-43FX Dye and Immunohistochemistry Staining

To visualize the neuromasts, FM1-43FX stain and fluorescent microscopy were used in combination with S100 stain and confocal microscopy. FM1-43FX, MS-222, PFA, and PBS were made as previously described in Chapter 3. After staining larvae with

FM1-43FX and fixing in PFA, whole larvae were stored in darkness at 4 ºC in PBS. Specimens were viewed within one week under a Leica MZ FLIII microscope equipped with epifluorescence. Neuromasts were counted and scored as either bright or faded. FM1-43FX emitted green fluorescence and required a blue excitation filter.

Immunohistochemistry was used to stain neuromasts, taste receptor cells, olfactory cells, and kinocilia. Larvae were euthanized and placed directly in PBS-T overnight at 4 ˚C. Larvae were then incubated with two primary antibodies diluted in PBS-T. S100 synaptic marker specific for olfactory cells and neuromasts and calretinin, specific for taste buds, olfactory cells, and the lateral line nerve were used. The same procedure was followed as previously described in Chapter 3. In all cases, the same two secondary antibodies were used; goat anti-rabbit and goat anti-mouse. Samples were incubated 96 hours, given three, 60 minute washes in PBS, then stored in PBS overnight and viewed initially with a Leica DM4000 B microscope equipped with epifluorescence. Blue and green excitation filters were used to visualize 488 nm fluorophores as green and 555 nm fluorophores as red, respectively. Selected preparations of neuromasts (posterior to the eye and on the trunk), taste buds, and nerves were then viewed and photographed using a Zeiss LSM 510 META laser scanning confocal microscope. In some cases, the lower jaw was isolated and viewed separately to photograph taste buds and taste buds on the upper jaw were photographed in the absence of the lower jaw.

4.2.3 Behavioural Observations Following Neomycin Treatment

To determine if neomycin affected swimming ability, larvae were observed using video recordings (Fig. 4.2; Lumix, Panasonic DMC-LX3 camera). Larvae (10 and 20 dph) were placed in 2 l plastic containers. At 10 dph, swimming ability was observed for controls (n=7), 2 mM neomycin treated larvae (n=7), and 5 mM neomycin treated larvae (n=7) at 30 minutes and two hours after neomycin treatment. At 160 minutes following neomycin treatment, the side of each 2 l holding container was tapped in three sets (2 to 4 taps per set) using metal forceps (10 cm length) with 4-6 second intervals between taps. A response was defined as an acceleration, change in direction, or an escape response (C-start). The number of larvae making a response was averaged across the number of taps.

At 20 dph, swimming ability was observed for neomycin treated larvae (n=5) given the highest dose tested (5 mM) and for one control. Stressed larvae (n=3) that had been transferred from one rearing tank to another that day were used as a secondary control for the purposes of video recording only. One additional, solitary set of taps (3 to 4) was given to each container ten minutes later. As these tests were purely observational with no experimental design, no statistical analysis was performed.

Figure 4.2 Experimental set-up for observing swimming of control and neomycin treated larval striped bass and responses to taps made with metal forceps.

4.2.4 Experimental Design and Statistical Methods

In the neuromast ablation trials, a split-plot ANOVA was used to analyze 2010 data because randomization of the run order of larval age (10 to 20 dph) was not possible. The whole plot treatment was age with levels 10, 13, 17, and 20 dph, the subplot treatment was concentration with levels 0, 1, 2, and 5 mM neomycin. The block consisted of three replicate strainers for each concentration and age. The experimental unit was one cell strainer containing four fish. All replicates within each age group were from two cohorts. The two response variables were the average number of total visible neuromasts on the lateral line and the number bright neuromasts only. When examining the larvae, trunks and tails that were damaged were not included in the averages. However, for each treatment combination at least nine samples out of the twelve were able to be averaged.

4.3 Results

4.3.1 Neuromast Ablation

Staining with FM1-43FX (3 replicates, 4 larvae per replicate) provided similar results as staining with S100 (n=1 to 3 larvae per treatment) as striped bass larval neuromasts were observed in similar numbers and locations (Table 4.1). Neuromasts treated with neomycin and stained with FM1-43FX appeared faded compared to controls at all ages. One example is shown at 10 dph (Fig. 4.3). At this age, the number of visible neuromasts of neomycin treated larvae (n=12) was similar to controls ($p>0.05$) regardless of dose, but neuromasts still appeared fainter indicatative of potential hair cell damage (Fig. 4.3). At 17 dph, neuromasts were faded, but visible on the trunk and tail when treated with 5 mM neomycin (Fig. 4.4). Specifically, the trunk of the control (A) shows eight visible neuromasts, while the neomycin treated trunk (B) shows only five visible neuromasts (Fig. 4.4). On the tail of the control (C) there are 15 neuromasts visible, while on the neomycin treated tail (D) there are only 12 visible.

Loss of S100 staining for cephalic neuromasts of a 14 dph larva treated with neomycin, located posterior to the eye was shown compared to a 13 dph control larva (Fig. 4.5; A and B). Larval neuromasts treated with neomycin appeared fainter in some cases, as on the trunk, while other were completely absent, as on the head (Fig. 4.5; A and B). On the trunk of a 14 dph neomycin treated larva, S100 stained neuromasts were visible, but staining was reduced compared to trunk neuromasts of similar position on a 13 dph control larva (Fig. 4.5; C and D). For both methods of staining, the infraorbital and supraorbital neuromasts were most affected by neomycin treatment (Appendix Table A.2).

Table 4.1 Average number of neuromasts around the eye and on the tail of S100 and FM1-43FX stained striped bass larvae age 10 to 20 days post hatch (dph).

	Eye		Tail	
Age (dph)	**S100**	**FM1-43FX**	**S100**	**FM1-43FX**
10	11 (n=1)	7.5 (n=12)	3 (n=1)	3.4 (n=12)
13	13.7 (n=3)	9.5 (n=12)	8.5 (n=2)	2.9 (n=12)
17	13.7 (n=3)	13.1 (n=11)	16.5 (n=2)	11.5 (n=11)
20	11 (n=1; canals forming)	12.4 (n=9)	18 (n=1)	16.3 (n=9)

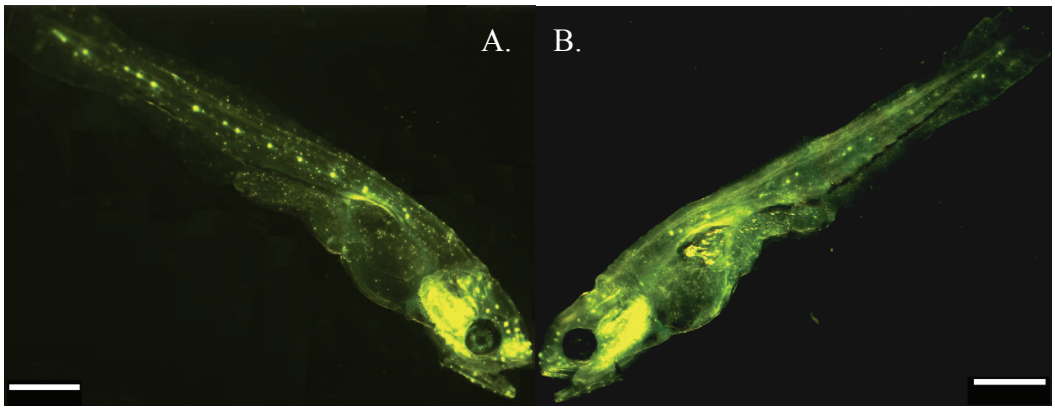

Figure 4.3 A. 10 dph control striped bass larva stained with FM1-43FX showing neuromast distribution B. 10 dph 5 mM neomycin treated striped bass larva stained with FM1-43FX showing many neuromasts visible, but faded. Both scale bars are 1 mm.

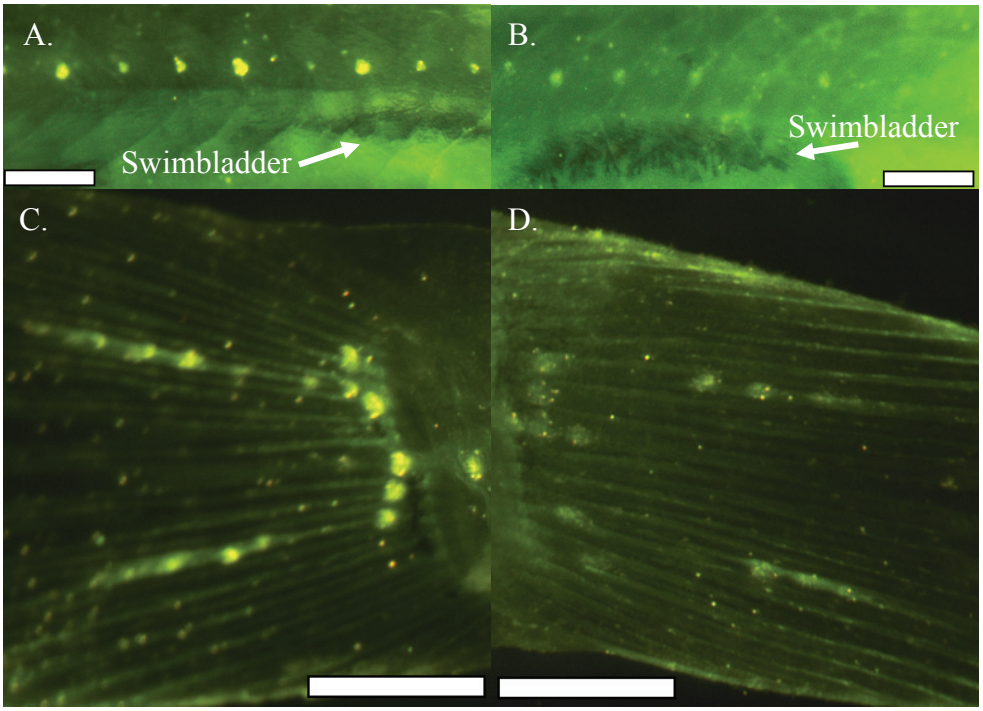

Figure 4.4 A. 17 dph control striped bass larva section of trunk above the swimbladder (arrow) showing 8 neuromasts as bright green dots stained with FM1-43FX and viewed with fluorescent microscopy. B. 17 dph striped bass larva treated with 5 mM neomycin showing 5 faded neuromasts on a similar section of trunk above the swimbladder (arrow) stained with FM1-43FX. C. 17 dph control striped bass larva tail showing 15 neuromasts stained with FM1-43FX. D. 17 dph striped bass larva tail neuromasts treated with 5 mM neomycin showing 12 neuromasts stained with FM1-43FX. All scale bars are 0.5 mm.

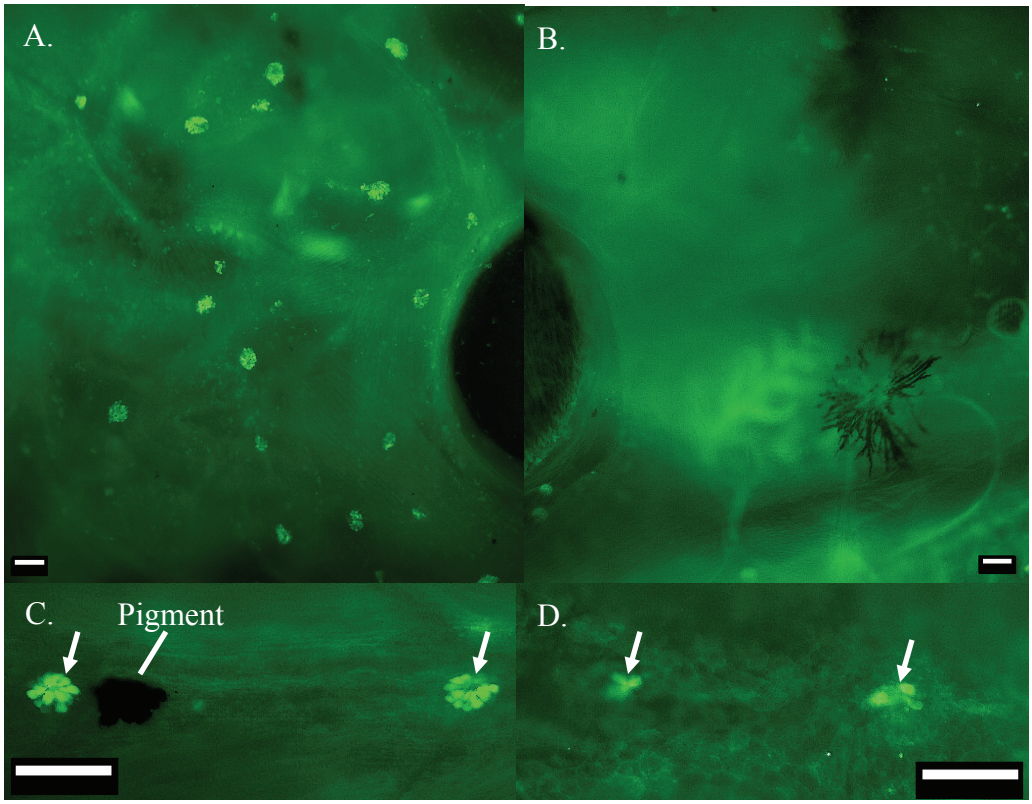

Figure 4.5 A. 13 dph control striped bass larva stained with S100 showing 19 neuromasts posterior to the eye. B. 14 dph striped bass larva treated with 5 mM neomycin for 60 minutes and stained with S100 showing complete loss of staining posterior to the eye. C. 13 dph control striped bass larva stained with S100 showing two neuromasts on the trunk (arrows) D. 14 dph larva treated with 5 mM neomycin and stained with S100 showing two faded neuromasts on the trunk (arrows). All scale bars are 100 μm.

Confocal microscopy provided more detail for determining whether neuromasts were damaged by neomycin. The lack of visible neuromasts on 14 dph neomycin treated larvae resulted in a small number of confocal images (two) showing damaged neuromasts on the head and trunk and therefore the equivalent number of images was used for 13 dph controls for comparison. Differences in neuromast structure with neomycin treatment were the loss of hair cells and darker appearance of damaged neuromasts. One 14 dph 5 mM neomycin treated larva (n=1 cephalic and 1 trunk neuromast) was compared with one 13 dph control larva (n=1 cephalic and 1 trunk neuromast; Fig. 4.6 A and B). The 13 dph control cephalic neuromast had twenty-seven hair cells while only nine hair cells were visible of the 14 dph neomycin treated cephalic neuromast from a similar position which also had missing hair cells, and was darker than the control neuromast. Cephalic neuromast hair cell survival rate from a single fish as a percentage of the total hair cells

of the control was 33% (9/27x100). The 13 dph control trunk neuromast had twelve hair cells, while the 14 dph 5 mM neomycin treated trunk neuromast had seven hair cells with some missing. (Fig. 4.7 A and B). The trunk neuromast hair cell survival rate from a single fish as a percentage of the total hair cells of the control was 58% (7/12x100).

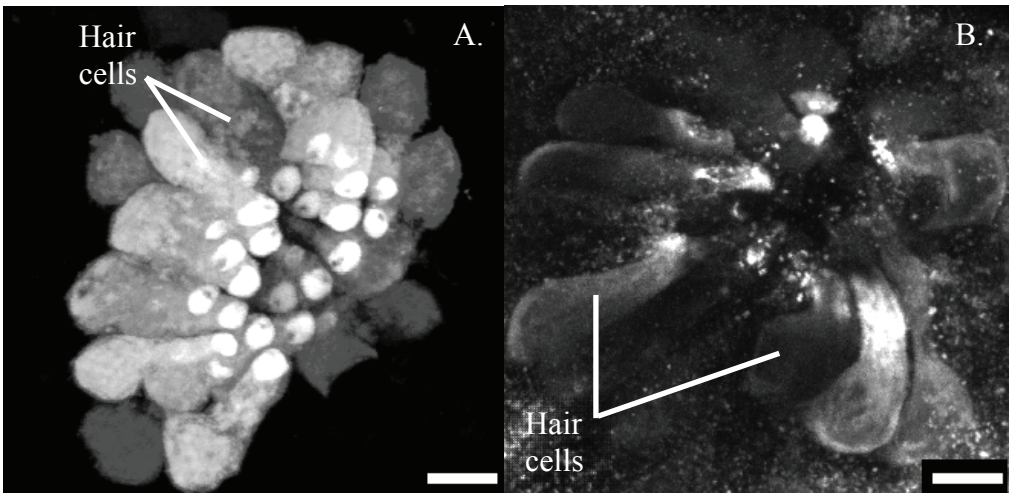

Figure 4.6 A. Cephalic neuromast stained with S100 located posterior to the eye of a 13 dph striped bass larva having 27 hair cells (Neuromast length: 33.6 μm, width: 25 μm, height: 21 μm). B. Cephalic neuromast stained with S100 in the same location posterior to the eye on a 14 dph striped bass larva treated with 5 mM neomycin for 60 minutes having 9 hair cells visible (Neuromast length: 34.7 μm, width: 25 μm, height: 14 μm). Both scale bars are 5 μm.

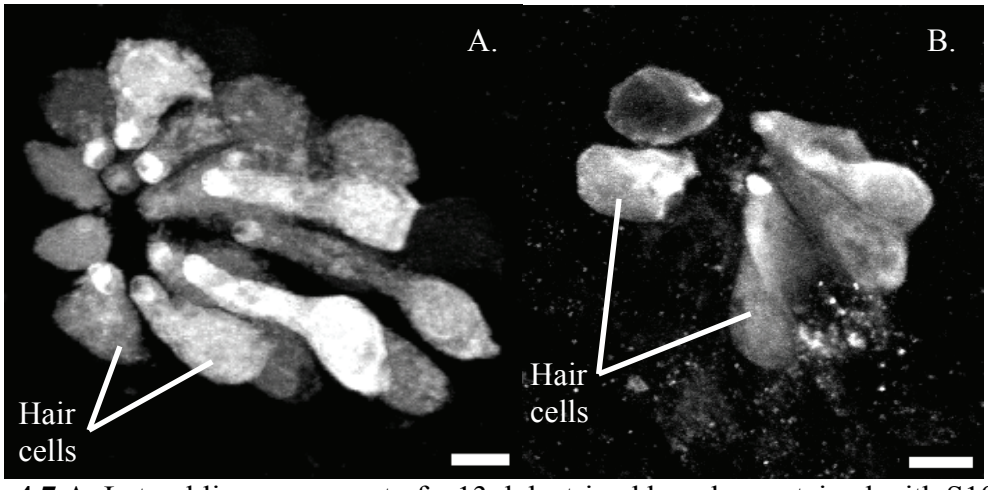

Figure 4.7 A. Lateral line neuromast of a 13 dph striped bass larva stained with S100 having 12 hair cells (Neuromast length: 37 μm, width: 22 μm, height: 21 μm) B. Lateral line neuromast of a 14 dph striped bass larva treated with 5 mM neomycin for 60 minutes and stained with S100 having 7 hair cells visible (Neuromast length: 26 μm, width: 17 μm, height: 13 μm). Scale bars are 5 μm.

The statistical analysis performed using the total number of visible neuromasts including bright and faded neuromasts as the response variable resulted in a significant interaction between age and neomycin dose (Table 4.2). Among control larvae the mean number of neuromasts increased significantly with age, from 28 at 10 dph to 90 at 20 dph (Fig. 4.8). Total mean body lengths ranged from 7.5 mm at 10 dph to 13.7 mm at 20 dph. Among larvae age 10 and 13 dph, increasing neomycin dose resulted in a decrease in neuromast visibility and number, but the effect was not significant (Fig. 4.8). By contrast, among larvae age 17 and 20 dph, all the doses of neomycin significantly reduced the mean number of visible neuromasts compared to controls (Fig. 4.8; Appendix Table A.3). A significant interaction was solely due to the data point of 5 mM neomycin treated larvae at 17 dph having a higher mean of visible neuromasts than the data point at the same treatment level at 20 dph.

Table 4.2 ANOVA p-values for mean total visible neuromasts of striped bass larvae at four developmental ages (10, 13, 17, 20 dph) and four neomycin doses (0, 1, 2, 5 mM), df = degrees of freedom.

	Mean total visible neuromasts	
Source of variation	**P**	**df**
Dose	<0.0001	3, 24
Age	<0.0001	3, 8
Age*Dose	<0.0001	9, 24

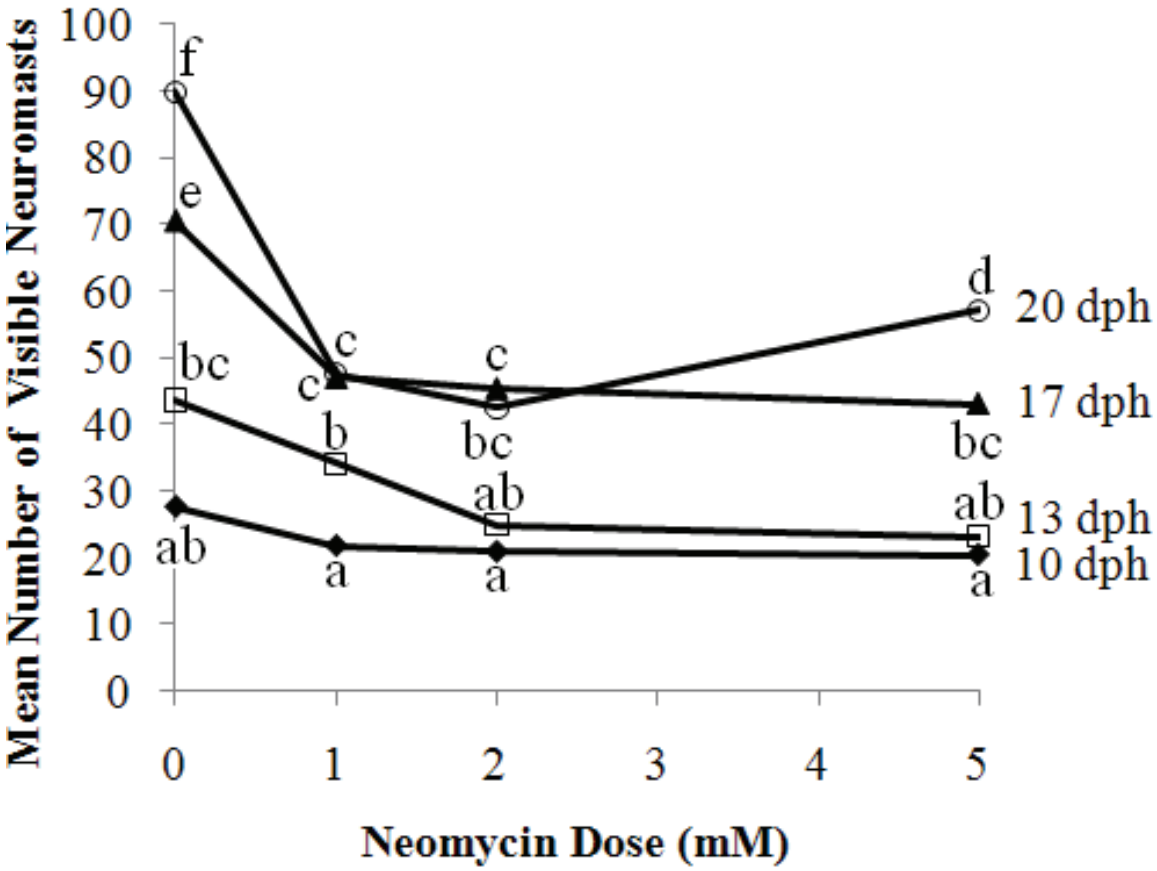

Figure 4.8 Mean number of visible neuromasts (bright and faded) on neomycin treated and untreated striped bass larvae stained with FM1-43FX dye under a fluorescent microscope. For each neomycin dose and age combination, means sharing the same letter are not significantly different at the 5% level.

By comparison, the statistical analysis performed using only the bright number of neuromasts as the response variable still resulted in a significant interaction between age and neomycin dose (Table 4.3). Among control larvae the mean number of bright neuromasts increased significantly with age, from 28 at 10 dph to 90 at 20 dph (Fig. 4.9). Among all larval ages tested, increasing neomycin dose resulted in a significant decrease in neuromast visibility and number (Fig. 4.9). The number of bright neuromasts was not significantly reduced following a dose of 1 mM neomycin, but was significantly reduced following doses of 2 and 5 mM neomycin (Fig. 4.9; Appendix Table A.4).

Table 4.3 ANOVA p-values for mean number of bright neuromasts of striped bass larvae at four developmental ages (10, 13, 17, 20 dph) and four neomycin doses (0, 1, 2, 5 mM), df = degrees of freedom.

	Mean total visible neuromasts	
Source of Variation	**P**	**df**
Dose	<0.0001	3, 24
Age	<0.0001	3, 8
Age*Dose	0.0003	9, 24

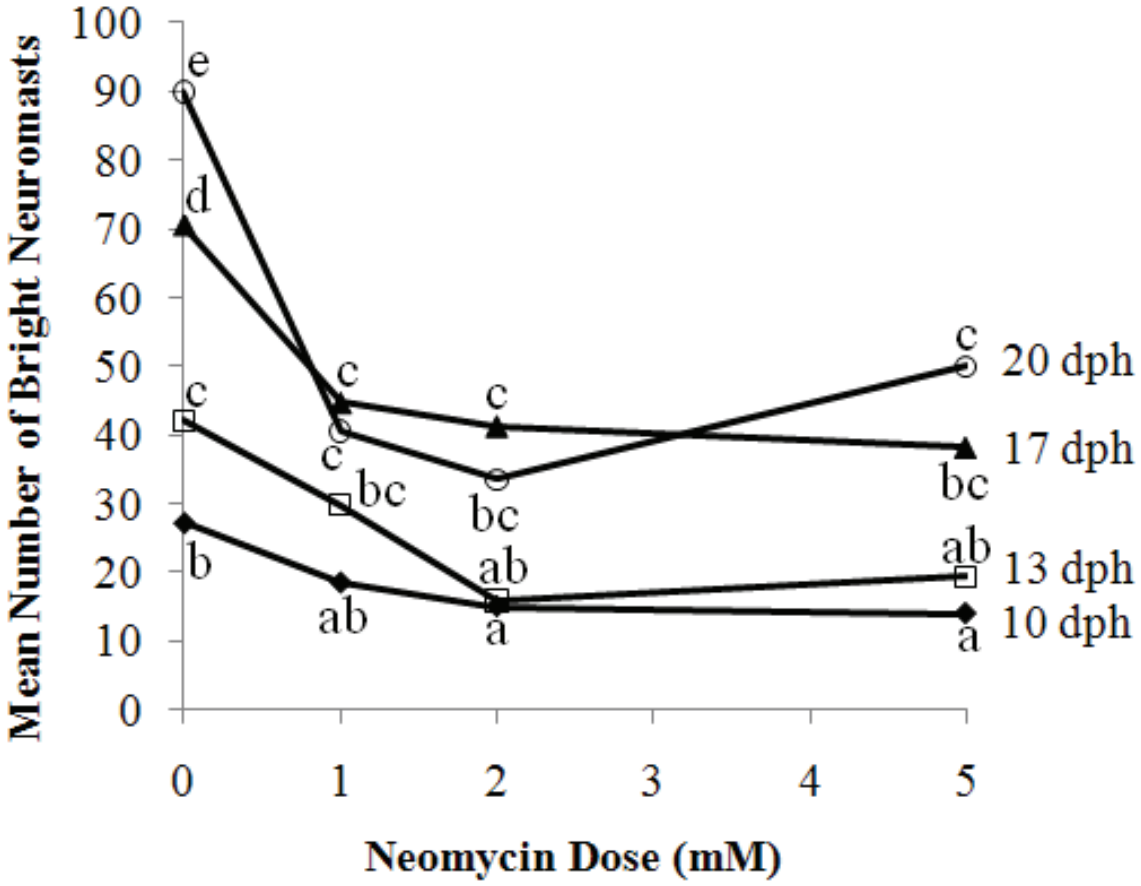

Figure 4.9 Mean number of bright neuromasts on neomycin treated and untreated striped bass larvae stained with FM1-43FX dye under a fluorescent microscope. For each neomycin dose and age combination, means sharing the same letter are not significantly different at the 5% level.

Even with neomycin treatment, the lateral line nerve was observed intact. Afferent nerve fibre projections on the tail (Fig. 4.10 A) treated with neomycin and stained with calretinin were observed in the same position as neuromasts stained with S100 on the same tail (Fig. 4.10 B).

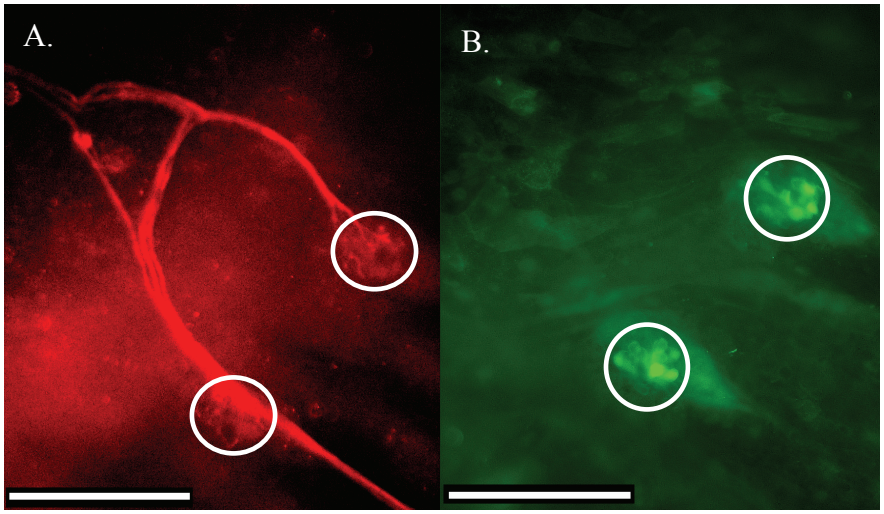

Figure 4.10 A. Lateral line nerve extending to the tail of a 14 dph striped bass larva treated with 5 mM neomycin and stained with calretinin. Circles indicate nerve endings where neuromasts are located and innervated. B. Damaged neuromasts (circles) correspond to the position of underlying nerve endings on the tail of the same larva stained with S100. Both scale bars are 100 μm.

Calretinin staining of control and neomycin treated specimens resulted in no apparent damage of taste or olfactory cells. Several striped bass larvae specimens of various ages were examined for the presence of taste buds including two 11 dph controls, one 14 dph neomycin treated larva, two 19 dph neomycin treated larvae, and two 20 dph neomycin treated larvae. There were no differences in the number of taste buds on the 20 dph control (12 taste buds) compared to the 19 dph 5 mM treated larva (12 taste buds) along the upper lip on one side with the lower jaw cut away (Fig. 4.11 A and B; Table 4.4). The olfactory pits of these two larvae were also visible indicating no damage to the sensory cells.

The 14 dph neomycin treated larva had more taste buds (9 upper, 10 lower) than the 11 dph controls (average: 7 upper, 5.5 lower; Table 4.4). At a higher magnification, two taste buds from the lower lip were examined for the 19 dph neomycin treated larva and one for the 20 dph control larva (Fig. 4.12). Taste buds were similar in size and

structure. However, the images were taken of a lateral view only and so the number of cells in each was estimated to be six.

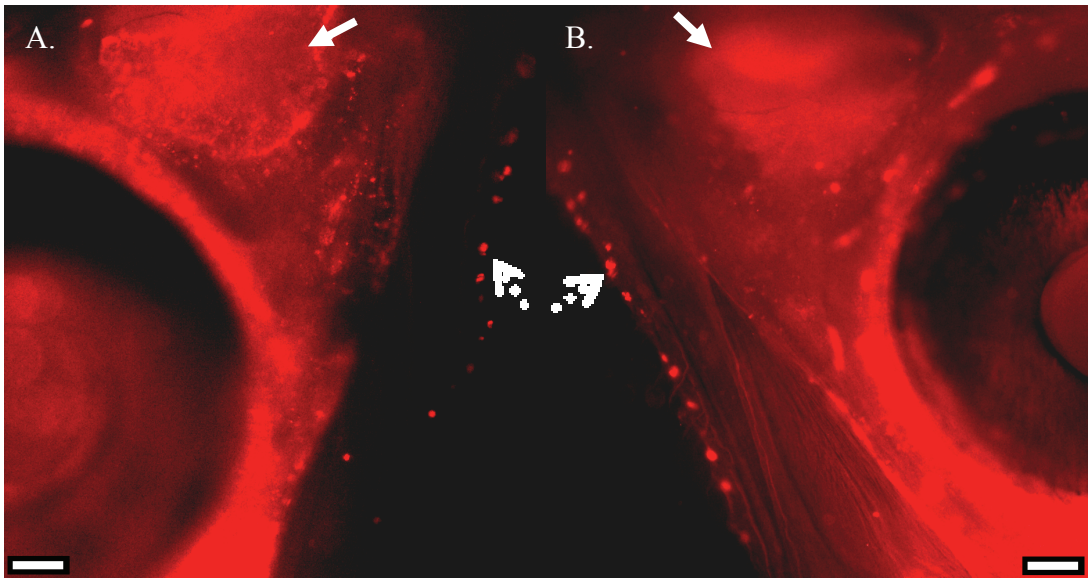

Figure 4.11 A. Upper jaw of a 19 dph striped bass larva treated with 5 mM neomycin and stained with calretinin having 12 taste cells visible on the lip (dotted arrow). The olfactory pit was also stained (solid arrow).B. Upper jaw of a 20 dph control striped bass larva stained with calretinin also having 12 taste buds on the lip (dotted arrow). The olfactory pit was also stained (solid arrow). Both scale bars are 100 µm.

Table 4.4 Average number of taste buds (range) on the lip of striped bass larvae age 11, 14, 19, and 20 dph. Taste buds on the lower lip were not counted for 19 and 20 dph because the jaw was cut away. n=number of larvae observed.

	Average number of taste buds		
Age (dph)	**Upper**	**Lower**	**n**
11	7 (6-8)	5.5 (4-7)	2
14	9	10	1
19	11.5 (11-12)	---	2
20	11.5 (11-12)	---	2

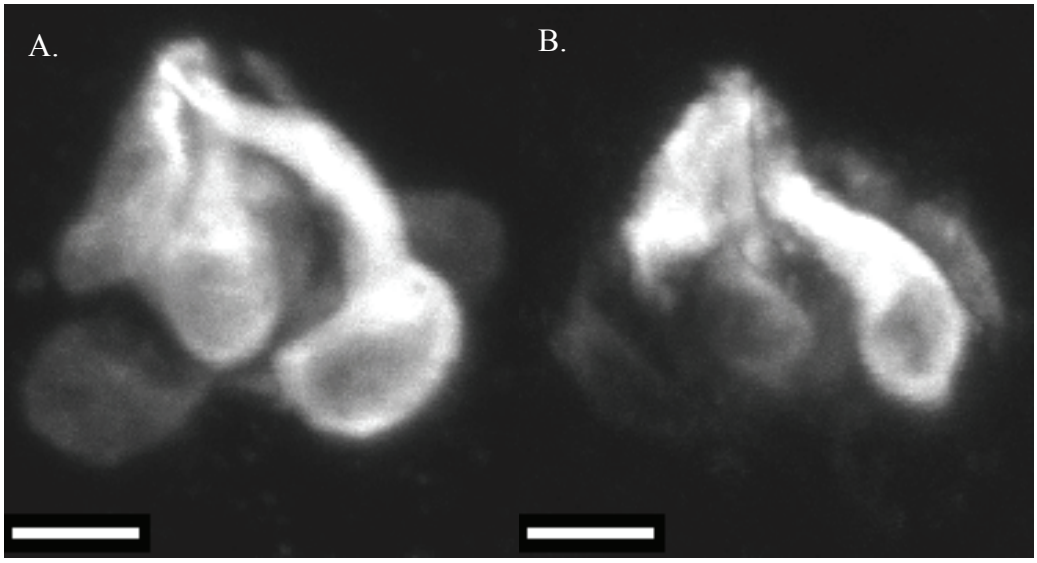

Figure 4.12 A. Taste bud from a 19 dph striped bass larva treated with 5 mM neomycin and stained with calretinin (Height: 14 μm, width:18 μm at widest part) B. Taste bud from a 20 dph control striped bass larva stained with S100 (Height: 11 μm, width: 19 μm). Both scale bars are 5 μm.

4.3.2 Swimming Behaviour Following Neomycin Treatment

To aid in selecting a neomycin dose that would not inhibit normal swimming ability, preliminary observations were made for swimming behaviour. Thirty minutes after neomycin treatment, swimming behaviour was normal for most 10 dph larvae treated with 2 or 5 mM neomycin when compared to controls. Normal behaviour of larvae was defined as actively swimming around the container in an upright position. However, a percentage of neomycin treated larvae were motionless following treatments with 14% of 5 mM treated larvae inactive and 57% of 2 mM treated larvae inactive (Table 4.5; Appendix Table A.5). Two hours after neomycin treatment, the same number of larvae was active in each condition with only one larva occasionally spinning after 5 mM neomycin treatment.

Tapping the container resulted in an average of 63% of 10 dph control larvae, 47% of 2 mM treated larvae and 33% of 5 mM treated larvae responding with an acceleration, change in direction, or escape response. Even though fewer responses were made on average by 5 mM neomycin treated larvae than by larvae in other groups, all but one were actively swimming.

At 20 dph, tapping resulted in 69% of control larvae, 73.3% of stressed controls (moved from tank to tank), and 21% of 5 mM neomycin treated larvae responding two hours and twenty minutes after neomycin exposure (Table 4.5; Appendix Table A.6). Ten minutes later, the one control larva and 90% of the stressed controls responded to taps, while only 6% of 5 mM neomycin treated larvae responded. In all cases, the controls consistently responded more frequently to the taps than the 5 mM neomycin treated larvae.

Table 4.5 Average number of striped bass larvae responding to taps on the side of a container at 10 and 20 dph at different times after neomycin treatment and percentage of larvae swimming normally (active and upright).

Age (dph), time following neomycin treatment (h)	**Dose (mM)**	**Average number of responses to a series of taps out of number active (± SD)**	**Percent responding out of number active (% ± SD)**	**Percent active (%)**
10, 2 h	Control	3.8/6 (± 0.50)	63 (± 12)	86 (6/7)
	2 mM	1.4/3 (± 0.22)	47 (± 16)	43 (3/7)
	5 mM	2.3/7 (± 0.85)	33 (± 15)	100 (7/7)
20, 2 h 20 min	Control	0.69/1 (± 0.37)	69 (± 41)	100 (1/1)
	Stress controls	2.2/3 (± 0.71)	73.3 (± 25)	100 (3/3)
	5 mM	0.83/4 (± 0.70)	21 (± 66)	80 (4/5)
20 dph, 2 ½ h	Control	1/1 (± 0.0)	100 (± 0)	100
	Stressed controls	2.7/3 (± 0.54)	90 (± 16)	100
	5 mM	0.25/4 (± 0.78)	6 (± 11)	80 (4/5)

4.4 Discussion

The assessment of neuromast damage by neomycin using both conventional fluorescent and confocal microscopy provided reliable visualization. Staining in both cases was reduced, but finding an objective method of quantification was difficult. Imaging of olfactory cells and taste buds following neomycin treatment provided confidence that only neuromasts were affected, but had not been previously reported.

The quantification of invisible neuromasts was difficult due to individual variation. However, comparisons of total visible or bright neuromast counts indirectly accounted for reduced staining and invisible neuromasts. The effect of neomycin on striped bass neuromasts was only dose dependent for 10-13 dph larvae when the response variable was the mean number of bright neuromasts. Using this response variable, the first significant effect occurred with a 2 mM neomycin dose for 10 and 13 dph larvae, whereas 4-5 dpf zebrafish larval neuromasts responded to neomcyin with a strong dose response and required much lower doses (50 μM) for this effect to be significant (Harris *et al.*, 2003). Striped bass larvae age 17-20 dph had significant reduction in FM1-43FX staining for all neomycin doses tested, but did not exhibit a dose response for either response variable used. In one study on zebrafish larvae, hair cell death was complete 90 minutes after neomycin exposure regardless of dose or exposure time (Owens *et al.*, 2009). Therefore, as long as the neuromasts are given enough time to ablate, as in this thesis study (2 h), the dose response may disappear.

Judging the degree of damage for striped bass neuromasts in an objective manner was difficult. As this type of study has never been reported for striped bass, templates of neuromast distribution were constructed to score neuromasts as present or absent. One problem was that the templates did not take into account individual variation and so were not used for that purpose. Counting the number of bright neuromasts also resulted in subjectivity in deciding which neuromasts were faded or bright. However, this approach had been performed in other studies (Harris *et al.*, 2003; Murakami *et al.*, 2003). Counting the total number of visible neuromasts was less subjective, but significance started to be lost. To account for these types of subjectivity, many studies have used several staining techniques (FM1-43, FM1-43FX, DASPEI, DiAsp, and phalloidin) to improve reliability and strengthen their findings (Harris *et al.*, 2003; Hernandez *et al.*, 2006; Owens *et al.*, 2008; 2009). For this thesis study on striped bass larvae, neuromast damage was first observed with fluorescent microscopy and then confirmed with confocal imaging to gain objectivity and reliability. Striped bass neuromast hair cells were damaged, dark, and some were missing following neomycin treatment which was also observed in zebrafish neuromasts (Williams and Holder, 2000; Murakami *et al.*, 2003; Harris *et al.*, 2003; Owens *et al.*, 2007; 2008).

The neomycin dose of 5 mM for striped bass larvae age 13 and 14 dph resulted in 33% of the control hair cells from one cephalic neuromast being stained, and 58% of the control hair cells from one trunk neuromast being stained. For 4-5 dpf zebrafish larvae, increasing the neomycin dose from 250 to 500 μM resulted in decreased hair cell staining from 13% to only 4.6% of the average number of control hair cells stained (Harris *et al.*, 2003). The percentages for zebrafish hair cell death were lower, but the striped bass larvae were older, a different species, different staing methods were employed, as well as different neomycin doses. Also, the percentages for striped bass larvae were not based on average hair cell counts because of the limited number of photographs showing neuromasts visible on neomycin treated larvae stained with S100.

Individual stains can act differentially on the same neuromasts also stressing the importance of using several stains for any study. In one study on larval zebrafish, neuromasts were stained using three methods, DASPEI, phalloidin, and tubulin, of which DASPEI slightly underestimated hair cell survival (defined as the number of hair cells stained) compared to phalloidin and tubulin (Harris *et al.*, 2003). However, similar trends were observed between the three stains in hair cell counts and 100 % elimination of staining was never observed, even at the highest neomycin dose. While all three indicators may have yielded different estimates of hair cell death, together they provided a strong indication that neuromasts were profoundly affected by neomycin. The use of both FM1-43FX and S100 staining methods in this thesis study provided two ways of quantifying hair cell death; one the counting of visibly stained neuromasts, and the other the counting of stained hair cells within specific neuromasts. The results from both methods support the findings of Harris *et al.* (2003) that neomycin negatively affects neuromasts. Specifically, immersion in 5 mM of neomycin for 60 minutes followed by a two hour recovery interval effectively damaged the structure of striped bass larval neuromasts.

At the lower neomycin doses tested (0.007 to 0.7), the number of mean neuromasts was similar between treated and control larvae possibly due to the high calcium content of the seawater used. Calcium is known to compete with aminoglycosides for binding sites at the mechanotransduction channel (Schacht, 1986).

Reducing the seawater concentration to 1 ppt and increasing neomycin concentrations to 5 mM likely contributed to more effective ablation.

Calretinin antibody was discovered as an effective label for the lateral line nerve and its projections to neuromasts which suggests a practical use for further study involving neuromast quantification following ablation. Since each nerve projection indicated the presence of a neuromast and because the nerve projections remained undamaged with neomycin treatment, the projections could be used as an indicator of neuromast location after ablation. The calretinin antibody has also been used to label other nervous structures having calretinin as a regulator of intracellular calcium, such as the grey mullet (*Chelon labrosus*) teleost brain sensory fibers and the zebrafish peripheral nervous system (Díaz-Regueira and Anadón, 2000; Levanti *et al.*, 2008).

The staining of taste and olfactory cells following neomycin treatment is novel research. The taste and olfactory cells were undamaged even by the highest dose of neomycin treatment (5 mM) as indicated by fluorescent and confocal images. After treatment with neomycin, these structures were still stained brightly indicating that calretinin, located in taste and olfactory cells, and S100 protein, located in olfactory cells, were still present which was indicative of normal cellular function. In contrast, neomycin treated neuromasts were faded and damaged indicating non-functionality due to damaged S100 protein located in neuromasts. Therefore, even though S100 protein is located in both olfactory cells and neuromasts, only neuromasts were damaged by neomycin, while taste and olfaction remained intact. Olfactory and taste buds in larval zebrafish, striped trumpeter, and willow shiner have been stained to show sensory development, but not after aminoglycoside treatment to determine possible damage (Alexandre and Ghysen, 1999; Cobcroft and Pankhurst, 2003; Mukai, 2006). This thesis study on striped bass larvae is the first to report potential effects of neomycin on taste and olfaction.

Previous studies treating larvae with streptomycin for feeding trials did not record observations of larval swimming behaviour before beginning the feeding trials (Batty and Hoyt, 1995; Cobcroft and Pankhurst, 2003; Mukai, 2006). Swimming behaviour at both 10 and 20 dph was observed to be normal for most striped bass larvae, even after structural neuromast damage using a 5 mM neomycin dose. However, responses to taps was lower for the neomycin treated larvae than controls, indicating the possibility of

dysfunctional neuromasts. Therefore, a dose of 5 mM neomycin was chosen to treat striped bass larvae for feeding trials due to evidence of ablation, the consistency between confocal and fluorescent microscopy using two different stains (FM1-43FX and S100), and normal swimming ability compared to controls. Neomycin caused structural damage to neuromasts, but whether neomycin would affect non-visual feeding was further investigated in Chapter 5 by quantifying prey capture.

Chapter 5 Role of Neuromasts in Non-visual Feeding

5.1 Introduction

Striped bass larvae native to the highly turbid Shubenacadie River estuary are able to feed in conditions of low visual acuity and are known to feed in the dark (McHugh and Heidinger, 1977; Chesney, 1989; MacIntosh and Duston, 2007). A strong selective pressure to feed in turbid conditions and extend feeding into the night may have led to the adapted ability of non-visual feeding (Chesney, 1989). The objective of this thesis was to test whether mechanoreception involving neuromasts of the lateral line system was involved in non-visual feeding. Ablation techniques have been used to investigate the role of neuromasts in non-visual feeding. Only four previous studies used ablation of both superficial and canal neuromasts to investigate the role of these mechanoreceptors in non-visual feeding in fish (Jones and Janssen, 1992; Batty and Hoyt, 1995; Cobcroft and Pankhurst, 2003; Mukai, 2006). However, none of these studies determined whether neuromasts were effectively damaged by the aminoglycoside or if other senses were affected as could be done through imaging. All four studies used feeding in the light as a control. Larval mottled sculpin (*Cottus bairdi*) tested with live *A. salina* prey were found unable to feed in the dark with superficial neuromast ablation by 0.05 mg l^{-1} streptomycin, unless directly touched by the prey (Jones and Janssen, 1992). Streptomycin used at 10 mM for 20 minutes inhibited juvenile sole (*Solea solea*) from feeding on *Tigriopus* shrimp (250 to 2500 μm) in the dark suggesting that mechanoreception was relied on more than chemoreception (Batty and Hoyt, 1995). However, feeding of juvenile plaice (*Pleuronectes platessa*) in the dark was not inhibited by streptomycin and chemoreception was proposed as the feeding mechanism without evidence of lateral line ablation (Batty and Hoyt, 1995). This study quantified the number of bites and not the number of prey caught, and therefore the validity of feeding efficiency was also questionable. Larval striped trumpeter (*Latris lineata*) were treated with 10 mM streptomycin for 15 to 20 minutes, but as controls fed as poorly in the light as treated larvae in the dark, no conclusions could be drawn regarding the non-visual feeding mechanism (Cobcroft and Pankhurst, 2003). Streptomycin was also used to ablate neuromasts on larval willow shiner (*Gnathopogon elongatus caerulescens*) which

significantly reduced feeding in the dark from 10.6 to 0.8 *A. salina* 10 minutes^{-1} (Mukai, 2006). Streptomycin treated larvae were able to catch 11 prey on average in the light which was not significantly different than the untreated controls which caught 12.3 prey. This result indicates that streptomycin had not caused abnormal swimming behaviour, did not affect motivation of the larvae to feed, or affect capability of finding prey visually (Mukai, 2006). However, the use of streptomycin could have damaged taste or olfactory cells providing an alternative explanation for the reduced prey capture in the dark. As larvae ranged in age from 21-33 dph for these feeding trials, the nostrils and taste buds were well developed and their potential role in non-visual feeding was not ruled out (Mukai, 2006).

In these previous studies, with no tests implemented to determine whether or not the neuromasts were ablated, decreased feeding rates could have been the result of other factors, such as damaged taste or smell, abnormal swimming ability due to the drug, or other unknown factors. Testing both control and treated larvae feeding in the light ensures that prey capture is normal and unaffected by the drug.

Species inhabiting turbid environments, such as striped bass, may have well adapted lateral line systems to aid in prey detection, but there is no published literature describing both feeding trials and imaging of ablated neuromasts for any species of fish. Functional neuromasts were hypothesized to detect prey, but no measures were taken in previous studies to determine if the neuromasts were actually rendered dysfunctional providing weak evidence for neuromast-mediated prey capture. This chapter will test the hypothesis that striped bass larvae use neuromasts to feed in the dark, a condition similar to depths below 2 m in their natural habitat, using an effective neomycin dose of 5 mM determined in Chapter 4.

5.2 Methods

5.2.1 Prey Capture Experimental Procedures

The hypothesis that neomycin treated larvae would exhibit reduced prey capture in the dark was tested by conducting feeding trials to quantify prey capture at 10, 13, 17, and 20 dph for control and neomycin treated larvae in either the light or dark. Larvae

younger than 10 dph were not used due to their inability to survive neomycin treatment. Larvae older than 20 dph were not used in feeding trials because they were approaching the juvenile stage. Larvae were not fed after 1700 hours to ensure that no food was left in the gut for the trial the next day.

Baskets were used to transport larvae between a number of solutions. The baskets were constructed from PVC pipe (4.8 cm tall, 7.5 cm internal diameter). Nitex mesh (625 μm) was glued 1.5 cm from the bottom and a notch made on the bottom edge of the basket to let water pass underneath. Handles made of nylon ties were added on the side for ease of movement between solutions, all at room temperature in keeping with the striped bass rearing temperature (18 ˚C).

Feeding trial tanks and procedures followed MacIntosh and Duston (2007). Eight black matt 44 l polyethylene tanks (30 cm tall, 43 cm diameter) with a conical base (30º angle) were prepared by inserting rubber stoppers (1 cm tall, 3.6 cm diameter) and attaching aeration rings (10 cm diameter; Aquatic Ecosystems part #OD4) around a centre drain plug to provide upwelling current to maintain *A. salina* prey in suspension. Air flow to each tank was kept as low as possible (0.35 l/min). Each tank was filled with 24.5 l freshwater (FW) and 5 l SW for a total volume of 29.5 l at a salinity of 5 ppt, matching the salinity of the rearing tanks. A domestic fan heater kept both the air and water temperature in the sealed room at 20˚C. Tanks were made light-proof by securing black felt covers in place and trials were conducted in a light-proof room.

The light source for each tank was a broad-spectrum daylight fluorescent bulb (20 watt, Spiralux Vita-lite #1171LN), mounted in an inverted, black metal can (17 cm tall, 15.5 cm diameter) with a 10 cm diameter aperture at the base. Light intensity was adjusted with neutral density filters (Lee Filters, Andover, U.K.). The lights were suspended in each tank to a height of about 26 cm above the water surface, providing an average of 23.9 lx (range of 22.1 to 25.6 lx) at the water surface which falls within the best range for striped bass larval visual feeding (MacIntosh and Duston, 2007). Four replicate tanks were used for both the neomycin treated and untreated larvae. Light and dark trials were run on alternate days so that tanks of larvae feeding in the light would not affect tanks of larvae feeding in the dark and the room was too small to accomplish enough replicates in a single day.

The morning of a trial at 08:00 h, one basket was placed in a container holding rearing water which entered the baskets via their nitex base. Larvae were gently captured from the rearing tank using two flour sifters and transferred to the dimly lit preparation room (~200 lx). In the first trials, 50 larvae were counted into baskets immersed in rearing water. Baskets were then transferred to one of two shallow plastic containers (5 cm tall, 12.5 cm diameter) filled with either 150 ml of 5 mM neomycin or 1 ppt SW (final volume in basket: 97 ml) and left for 60 minutes. The baskets were given two 20 second rinses and a chase (recovery) interval of 2 hours in 1 ppt seawater. The larvae were then counted into eight separate containers, each containing 250 ml 1 ppt SW (15 larvae per container) and taken to the feeding trial room. As experiments proceeded, it was observed that approximately half the neomycin treated larvae were not consuming food. Therefore, to provide more data, the number of larvae per basket was increased to 72 (24 larvae per tank). Each tank was randomly assigned either the control or neomycin treated larvae. The larvae were left undisturbed for 60 minutes to acclimate, included in the two hour recovery period. The heater fan was turned off to provide a quiet room.

A concentrated suspension of stage I *A. salina* was held in a 2 l container with vigorous aeration and five 0.1 ml samples were taken and the number of *A. salina* counted. The counts were averaged and the required volume was calculated to obtain a final concentration of 200 *A. salina* l^{-1} for each of eight beakers. The final volume was 250 ml SW containing 6000 prey on average to be added to the 30 l of seawater in the tanks. Prey were evenly distributed in each of the eight tanks, at three minute intervals to stagger the end of the 60 minute feeding period, allowing time for tank draining and fish capture.

Two individuals were required to drain the tanks and two small flashlights with red filters were used as fish cannot see infra-red wavelengths (Blaxter, 1983). To drain each tank, a two-inch PVC drain pipe was fitted to the tank bottom, a nylon stocking was fitted over the opposite end, and the drain plug was pulled. To prevent larvae from getting stuck to the tank walls, the water was gently swirled by hand. Larvae were caught in the stocking as the water drained out. Water was collected in a 2 l pitcher to flush out larvae remaining in the tank. The stocking was then removed, tied off, and immersed in a

numbered container holding 100 ml ice-cold MS-222 (concentration 0.2 g l^{-1}) to euthanize the fish.

With the aid of a dissecting microscope, each larva was dissected and *A. salina* in the mouth, throat, or stomach were counted. Only larvae that fed were included in the results to account for the non-feeders. The two experimenters were blind to the treatment given to the larvae. The presence or absence of a swimbladder was recorded for all larvae along with the total length (TL) of the first fifteen. All experiments were approved by the NSAC Animal Care and Use Committee (2005), ACUC File #2010-019.

5.2.2 Experimental Design and Statistical Methods

The experimental design for prey capture rate was a 2x2 factorial repeated measures. The factors were light intensity: dark (0 lx) and light (22 lx), and treatment: control and 5 mM neomycin. The response variable was the average number of *A. salina* captured per tank, per hour excluding non-feeders. Four replicate tanks were used for the purpose of analysis. To satisfy the assumptions for the repeated measures ANOVA, a cubic root transformation was performed on the mean prey captured data. Least squares means (lsmeans) were computed for the 3 way interaction between light intensity, neomycin treatment, and age. Letter groupings were generated at the 5% level to indicate which means were significantly different.

The second response variable was feeding incidence: the percentage of larvae having caught one or more prey items. The CATMOD procedure was used to analyze the data with the generalized logits response function (SAS Institute Inc. 2003) represented by the equation:

$$\text{generalized logit} = \text{LOG}\,(p_i/p_k)$$

where p_i is the i^{th} cell probability and p_k is the last cell probability. Significant treatment effects were compared using the contrast statement of the CATMOD procedure. Contrasts were computed for all possible pairs of age and treatment combinations (light intensity and neomycin treatment). Letter groupings were generated at the 5% significance level.

5.3 Results

Prey capture by larval striped bass was significantly affected by all factors tested resulting in a three-way interaction between age, light intensity, and the 5 mM neomycin dose (Table 5.1). In the light, larvae treated with neomycin caught fewer prey than untreated controls in the light. However, this effect was only significant for larvae age 10 dph (Fig. 5.1). In the dark, neomycin treated larvae had a very low prey capture rate averaging 5.5 *A. salina* caught per hour and did not improve with age. Control larvae caught more prey in the dark than neomycin treated larvae and this improved with age from 16 prey h^{-1} at 10 dph to 72 prey h^{-1} at 20 dph (Fig. 5.1; Appendix Table A.7). Larvae grew from an average total length of 7.4 mm (range: 6-8.2 mm) at 10 dph to 11.9 mm (range: 9-14 mm) at 20 dph.

Table 5.1 ANOVA p-values for mean prey capture rate by striped bass larvae of *A. salina* per hour per larva by control and 5 mM neomycin treated larvae in either light (20 lx) or dark (0 lx) at 10, 13, 17, and 20 dph.

	Mean prey capture rate	
Source of variation	**p**	**d.f.**
Age	<0.0001	3, 48
Light intensity	<0.0001	1, 48
Age*Light intensity	<0.0001	3, 48
Neomycin	<0.0001	1, 48
Age*Neomycin	0.1488	3, 48
Light intensity*Neomycin	<0.0001	1, 48
Age*Light intensity*Neomycin	0.0006	3, 48

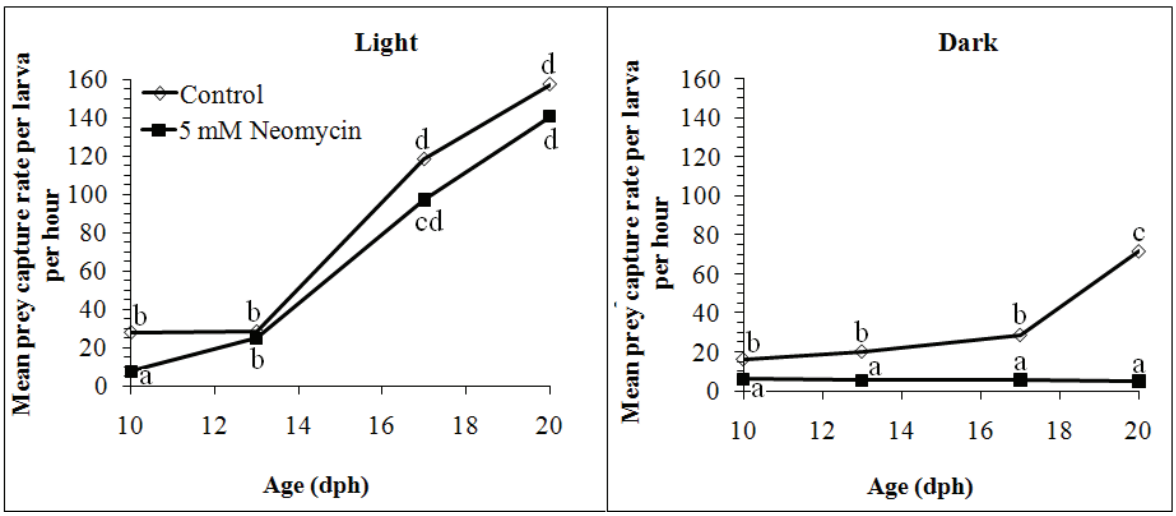

Figure 5.1 Mean prey capture rates of control and 5 mM neomycin treated striped bass larvae feeding in the light (20 lx) and dark (0 lx) at 10, 13, 17, and 20 days post hatch (dph). Data shown was from all fish that had caught at least one *A. salina* prey. Means sharing the same letter are not significantly different at the 5% level.

Feeding incidence was above 75% for control larvae feeding in the light and over 60% for control larvae feeding the dark (Fig. 5.2; Appendix Table A.8). More than 30% of 10 dph neomycin treated larvae fed in the light which increased to 62% at 20 dph. Neomycin treated larvae feeding in the dark had the lowest feeding incidence of 19% at 10 dph, which increased with age to 41% at 20 dph. Collapsing over age, the feeding incidence of larvae in each treatment was 86% light-control, 48% light-neomycin, 69% dark-control, and 35% dark-neomycin (Table 5.2). Overall, higher numbers of control larvae fed compared to neomycin treated larvae whether feeding in the light or dark, but feeding incidence never reached 100% for either treated larvae or untreated controls.

There was a difference in the average total lengths of larvae age 20 dph used in feeding trials (TL: 12 mm) and for neuromast ablation (TL: 14 mm). However, the mean number of neuromasts for four larvae from the feeding trial cohort was 85, while the mean for twelve controls from the neuromast ablation trials was 90, indicating no developmental difference concerning neuromast numbers.

Swimbladder inflation rate was noted as an aside with regards to feeding ability. The majority of non-feeders (65% on average) across all treatment groups were larvae without swimbladder inflation (Appendix A.9). However, some larvae without swimbladders were noted to feed in the dark suggesting that the swimbladder is not required for feeding.

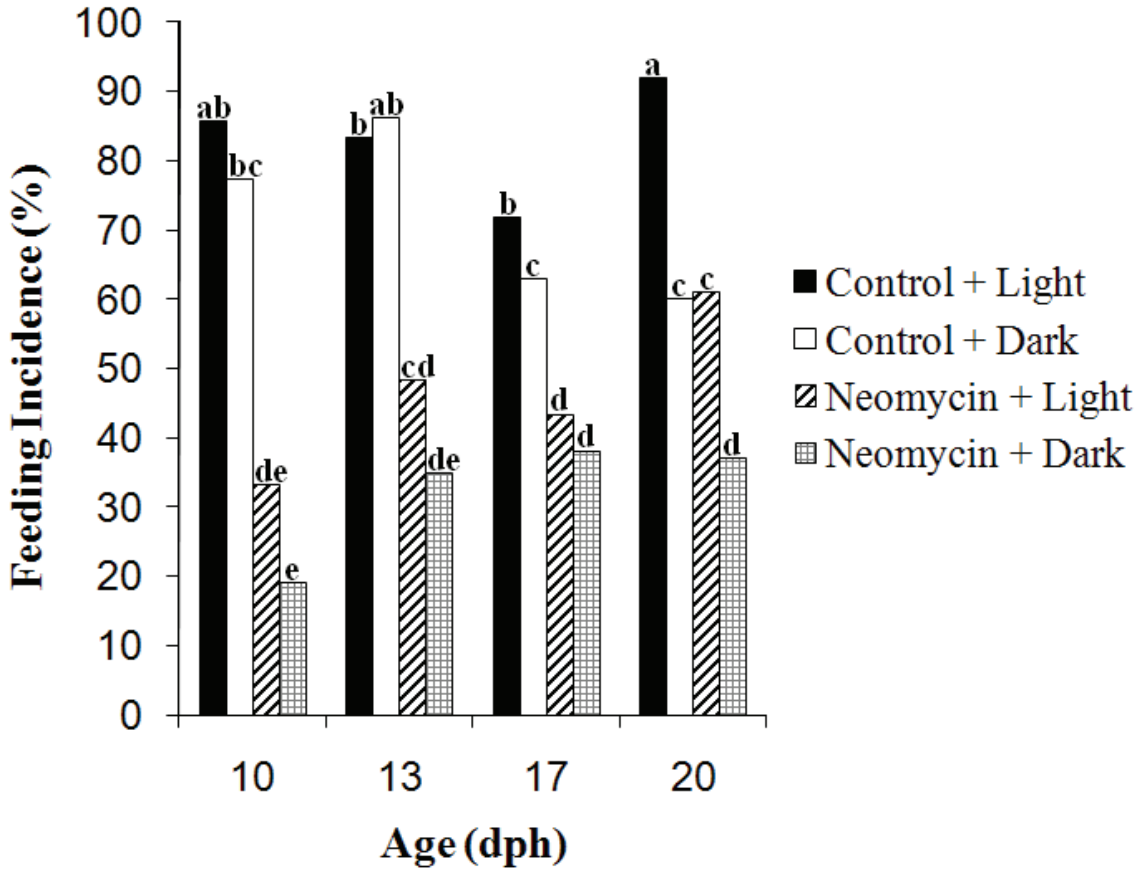

Figure 5.2 Feeding incidence of larval striped bass having caught one or more prey plotted against age of larvae (days post hatch) of four treatment combinations (control/light, control/dark, neomycin/light, neomycin/dark). Neomycin dose was 5 mM. For each larval age, histogram bars having the same letter are not significantly different at the 5% level.

Table 5.2 Percent feeding incidence of larval striped bass for each treatment combination (control/light, control/dark, neomycin/light, neomycin/dark) pooled across age. Neomycin dose was 5 mM.

Light-Control	Light-Neomycin	Dark-Control	Dark-Neomycin
86	48	69	35

5.4 Discussion

The reduction in prey capture ability of neomycin treated striped bass larvae in the dark provides evidence that neuromasts contribute to non-visual feeding. Only three

other accounts describe the phenomenon of non-visual feeding in striped bass larvae, but with no strong evidence of neuromast involvement (McHugh and Heidinger, 1977; Chesney, 1989; MacIntosh and Duston, 2007). Striped bass larvae were observed to grow when fed in complete darkness from 5 dph to 25 dph, but the number of *E. affinis* and *A. salina* caught at these ages was not quantified (Chesney, 1989). Control 10 dph striped bass larvae in this thesis were observed to feed in the dark at a rate of 16 *A. salina* larva^{-1} h^{-1}, whereas MacIntosh and Duston (2007) reported striped bass larvae ages 9 to 11 dph catching only 5 *A. salina* larva-1 h-1 in dim light. However, this difference was likely due to the lower prey density (100 *A. salina* l^{-1}) fed in the 2007 study compared to the 200 *A. salina* l^{-1} used in this thesis as well as the number of hours since the larvae last fed. In 2010 experiments, larvae were starved 6 hours longer than in 2007 experiments and may also provide explanation as to why the larvae feeding in the dark ate more in 2010.

The conclusion that striped bass larvae require functional neuromasts for non-visual feeding was supported only by one other study testing 11 parts per million (ppm) streptomycin for 24 hours on pre-metamorphosis willow shiner larvae (21 to 33 dph; 7.8 to 12.8 mm) which inhabit calm lakes (Mukai, 2006). Treated larvae caught comparable numbers of prey (11 *A. salina* 10 minutes^{-1}) to controls (12 *A. salina* 10 minutes^{-1}) in the light, but in the dark caught fewer prey (0.8 *A. salina* 10 minutes^{-1}) than controls (11 *A. salina* 10 minutes^{-1}).

Control striped bass larvae were observed to feed in the dark at a rate of 16 *A. salina* larva^{-1} h^{-1}, whereas in dim light, striped bass larvae ages 9 to 11 dph caught only 5 *A. salina* larva^{-1} h^{-1} as reported in MacIntosh and Duston (2007). However, this difference was likely due to the lower prey density (100 *A. salina* l^{-1}) fed in the 2007 study compared to the 200 *A. salina* l^{-1} used in this thesis as well as the number of hours since the larvae last fed. In 2010 experiments, larvae were starved 6 hours longer than in 2007 experiments and may also provide explanation as to why the larvae feeding in the dark ate more in 2010.

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The superficial neuromast cupulae of the willow shiner were longer than for other cyprinid fish, which is typical of species inhabiting calm waters where background noise levels are low (Engelmann *et al*., 2002; Mukai, 2006). Non-visual feeding via neuromasts seems important even for calm lake species, such as the willow shiner, indicating that neuromasts are used by a broader range of species than only those inhabiting turbid estuaries. If this is the case, mechanoreception would provide the ability for non-visual feeding at night, which is especially relevant during the larval and juvenile growth period between June and October. During this season, there are six hours of darkness at night and a critical size must be reached for improved chances of surviving the winter (Hurst and Conover, 1998).

Larval striped bass feeding ability compared between dark-controls (mechanoreception only) and light-neomycin (vision only) treatments showed that 10 dph larvae relied more on mechanoreception than vision, 13 dph larvae had equal reliance on both senses, and 17 dph larvae and older had a higher reliance on vision than mechanoreception. The reduction of prey capture in the dark was more evident for older larvae treated with neomycin which was possibly due to their stronger reliance on vision as the eye became more developed. In a predator avoidance experiment on larval Atlantic croaker (*Micropogonias undulates*), a comparison of dark-control and light-streptomycin treatments resulted in a shift towards vision at more than 6 mm TL (Poling and Fuiman, 1997). However, larvae less than 6 mm TL had equal reliance on vision and mechanoreception. Striped bass larvae older than 16 dph also demonstrated better prey capture ability in bright light than in dim light or darkness (MacIntosh and Duston, 2007). Mechanoreception seems to provide sensory information for early feeding striped bass larvae while the eyes are developing and potentially acts as backup when visual acuity is limited at depths below 0.5 m in the Shubenacadie River and at night (Chesney, 1989; Miner and Stein, 1993; MacIntosh and Duston, 2007).

These experiments were conducted in a lab where bubble rings were the only source of water disturbance besides the swimming invertebrate prey. Compared to the

turbulent Shubenacadie River, the feeding condition was relatively calm. One study investigating mottled sculpin discovered that hydrodynamic flow around the pectoral fins provided shelter to the trunk neuromasts from the ambient noise of the surrounding water (Coombs *et al.*, 2007). Furthermore, cephalic neuromasts were more densely distributed and had more hairs cells than trunk neuromasts which was also observed on larval striped bass in the present study (Janssen *et al.*, 1987). Therefore, it seems that larvae inhabiting turbulent waters are not compromised in their ability to use mechanoreceptors to detect minute vibrations of prey against a background of noise.

Larvae treated with neomycin exhibited a decrease in feeding incidence in the dark, but the percentage of feeders increased with age and stabilized at 20 dph. This increase in feeding incidence may have been due to improved swimming ability. However, neomycin treated larvae had a lower feeding incidence (41%) in the dark than controls (60%), even at the oldest age tested (20 dph). A comparison of control and neomycin treated larval feeding incidence in the light at 20 dph, showed a significant difference, 95% and 62% respectively, indicating that neomycin had a negative biological effect on the number of larvae able to feed. A decrease in feeding incidence was also observed for striped trumpeter larvae controls (27%) compared to streptomycin treated larvae feeding in the light (19%; Cobcroft and Pankhurst, 2003). However, feeding incidence was not reported for willow shiner larvae treated with streptomycin (Mukai *et al.*, 1994; Mukai, 2006). Even though the overall feeding incidence was low including controls, this result in concert with the current result for striped bass larvae may suggest that reduced feeding incidence is a side effect of aminoglycoside neuromast ablation. For this reason, only the striped bass larvae that caught at least one prey item were included in the analysis which was also done by Cobcroft and Pankhurst (2003). Some neomycin treated striped bass larvae appeared to swim normally, but a certain percentage of those were observed lying motionless, possibly because of ablated neuromasts. Therefore, neomycin may differentially affect individuals as the treated striped bass larvae that did feed caught the same amount of prey as controls. However, some uncertainty remains as to how neomycin impacts larval fish on the whole as no reports describe the side effects of aminoglycosides on fish.

To address the issue of potential sensory failure from neomycin treatment, previous staining and imaging of olfactory and taste cells was performed (chapter 4). Staining and imaging provided confidence that these senses were not affected by neomycin and therefore, were not confounding factors during feeding trials. Therefore, reduced feeding ability in the dark was not due to damaged taste or olfactory receptor cells. However, this is the first known documentation of taste bud and olfactory cell staining following neomycin treatment and damage may have occurred which was not detectable by the histological methods employed. Furthermore, reduced prey capture was not likely due to hearing loss from neomycin because external exposure to neomycin does not affect the hair cells of the otolithic organ as they are enclosed within the skull (Murakami *et al.*, 2003; Karlsen and Sand, 1987). Therefore, reduced prey capture in darkness following 5 mM neomycin treatment suggests the involvement of neuromasts in non-visual feeding.

Chapter 6 Conclusions and Future Research

The experiments conducted in this thesis provided the first description of larval neuromast ontogeny in striped bass and the first evidence that neuromasts contribute to non-visual feeding in these larvae. In addition, ablation of neuromasts did not inhibit larval feeding in the light. This suggests that neomycin is not affecting swimming or prey capture ability of larvae that fed, however feeding incidence was significantly reduced following neomycin treatment. The conclusion drawn from this study was that early stage larvae (10 dph) depend on mechanoreception more than vision, but as vision improves, the reliance on mechanoreception decreases. However, non-visual feeding potentially remains useful throughout life as adults are known to feed in the dark as well (Mueller and Horn, 1999). Furthermore, olfaction and gustation seemed to be unaffected by neomycin, although damage undetected by histology was possible. As taste and smell were intact for the feeding trials, these senses may account for the ability of neomycin treated larvae to feed at the low average rate of 5 A. salina $larva^{-1}$ $hour^{-1}$ in complete darkness. This hypothesis should be tested with the ablation of taste and smell for feeding trials conducted in complete darkness.

The lateral line was functional at 10 dph as indicated by similar prey capture rates in the light as in the dark by controls. Larvae only 3 days younger at the age of first feeding (7 dph) should be tested for the same requirement of neuromasts to accomplish initial feeding. However, concentrations of neomycin tested in this thesis were toxic for larvae younger than 10 dph. Therefore, further work is required to investigate concentrations suitable for young larvae to test the functionality of the lateral line at first feeding.

Furthermore, this study only investigated a lateral view of striped bass larvae. The trunk neuromasts followed a single lateral line, but further investigation could be done to determine possible neuromast arrangements on the dorsal-ventral plane.

The sense of mechanoreception may be useful for striped bass larvae feeding in a habitat where visual acuity is far less than for larvae feeding in clear water. Weakfish larvae (*Cynoscion regalis*) which inhabit clear water do not develop superficial neuromasts until 13 dph, but begin to feed at 8 dph suggesting that neuromasts may not be as important for this species (Connaughton *et al.*, 1994). At the other end of the

spectrum, neuromasts are abundant on the blind cavefish, indicating that superficial neuromasts contribute for feeding in darkness (Teyke, 1990). However, cavefish also make use of chemoreception to feed non-visually and likely works in concert with mechanoreception to achieve non-visual feeding.

In summary, recruitment of larvae to young-of-the-year is influenced by feeding success and growth. The pressure to maximize feed intake is facilitated by larval ability to extend feeding into the night. Data on feeding has emphasized visual feeding which dominates clear, oceanic waters. However, for turbid, estuarine environments, non-visual feeding involving mechanoreception is pertinent for survival and growth.

The sensory basis of feeding is complicated and depends on many modalities working together and providing backup when reliance on one modality is hampered as in the dark and turbid waters of the Shubenacadie. This study shows that neuromasts appear to contribute to such non-visual feeding.

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Appendix

Raw data tables

Table A.1 Description of striped bass neuromasts imaged at larval ages of 11, 13, 14, and 20 dph using confocal microscopy.

	Control + Head	5 mM + Head	Control + Trunk	5 mM + Trunk
11 dph	**n=3**			
Hair cell count	30			
Length (μm)	46			
Width (μm)	26			
Height (μm)	12			
13 dph	**n=1**		**n=1**	
Hair cell count	27		12	
Length (μm)	34		41	
Width (μm)	25		23	
Height (μm)	21		22	
14 dph		**n=1**		**n=1**
Hair cell count		9		7
Length (μm)		35		26
Width (μm)		25		17
Height (μm)		14		13
20 dph	**n=2**			
Hair cell count	NA			
Length (μm)	90			
Width (μm)	57			
Height (μm)	32			

Table A.2 Mean number of visible neuromasts at various locations on striped bass larvae from 10 to 20 dph (n = 12). POP/M=preopercular and middle, OP=opercular, P/O=posterior and occipital, Term=terminal, TV=total visible neuromasts.

10 dph	**Eye**	**Jaw**	**Nose**	**POP/M**	**OP**	**P/O**	**Term**	**TV**
Control	7.5	0.6	0	1.6	0	14.6	3.3	27.6
1mM	7.3	0	0.1	0.8	0	10.8	2.8	21.8
2 mM	5.8	0.4	0	0.7	0	10.6	3.2	20.7
5 mM	6.3	0.3	0	1.9	0	10	2.1	20.6
13 dph	**Eye**	**Jaw**	**Nose**	**POP/M**	**OP**	**P/O**	**Term**	**TV**
Control	9.5	0.9	0.2	2.6	0	27.8	2.9	43.5
1mM	7.4	0.6	0.2	2	0	22.5	1.6	34.1
2mM	5.4	0.3	0.1	2.1	0	15.2	2.4	25
5mM	4.4	0.3	0.1	1	0	15.5	1.4	22.7
17 dph	**Eye**	**Jaw**	**Nose**	**POP/M**	**OP**	**P/O**	**Term**	**TV**
Control	10.9	0.2	0.3	2.8	0.4	43.3	12.8	70.7
1mM	6.4	0.2	0.1	1.6	0	31.7	7	47
2mM	4.9	0.2	0	1.8	0	29.8	8.7	45.3
5mM	4.6	0	0	1.5	0	30.8	6.2	43.1
20 dph	**Eye**	**Jaw**	**Nose**	**POP/M**	**OP**	**P/O**	**Term**	**TV**
Control	12.3	0.4	0.2	5	1.1	52.8	18.1	89.5
1mM	6.3	0.3	0.3	2.2	0	30.4	8.1	47.6
2mM	3.6	0	0	0.4	0	30.5	7.9	42.4
5mM	4.5	0	0	0.4	0	41.6	10.7	57.1

Table A.3 Mean number of total visible neuromasts (SE) at four larval ages (10, 13, 17, 20 dph) and four neomycin doses (0, 1, 2, 5 mM). Means sharing the same letter are not significantly different at the 5% significance level.

Neomycin Dose (mM)	**Age (dph)**			
	10	**13**	**17**	**20**
0	^{ab}27.6 (1.0)	^{bc}43.6 (5.8)	^{e}70.6 (6.5)	^{f}89.9 (4.7)
1	^{a}21.8 (2.5)	^{b}34.1 (3.1)	^{c}47.0 (1.8)	^{c}47.6 (6.0)
2	^{a}20.9 (1.5)	^{ab}24.9 (3.0)	^{c}45.3 (2.1)	^{bc}42.4 (4.3)
5	^{a}20.4 (1.1)	^{ab}23.1 (7.7)	^{bc}43.0 (6.7)	^{d}57.1 (0.6)

Table A.4 Mean number of bright neuromasts (SE) at four larval ages (10, 13, 17, 20 dph) and four neomycin doses (0, 1, 2, 5 mM). Means sharing the same letter are not significantly different at the 5% significance level.

Neomycin Dose (mM)	Age (dph)			
	10	**13**	**17**	**20**
0	[b]27.3 (1.2)	[c]42.1 (6.7)	[d]70.6 (6.5)	[e]89.9 (4.7)
1	[ab]18.5 (1.9)	[bc]29.8 (6.2)	[c]44.7 (1.1)	[c]40.6 (6.2)
2	[a]15.0 (4.1)	[ab]15.9 (7.0)	[c]41.3 (1.1)	[bc]33.5 (1.3)
5	[a]14 (1.3)	[ab]19.4 (4.7)	[bc]38.2 (6.2)	[c]50.0 (2.9)

Table A.5 Raw data for the number of control and neomycin treated 10 dph larvae eliciting a startle response to tapping two hours after treatment.

		Number of Larvae Responding to Tapping			**Number of Larvae Active and Upright Out of Total**		
	Tap #	Control	2 mM	5 mM	Control	2 mM	5mM
First Set							
	1	5	2	1	6/7	3/7	7/7
	2	3	2	4			
	3		1	2			
	4			2			
Second Set							
	1	4	1	3	6/7	3/7	7/7
	2			3			
	3			2			
Third Set							
	1	3	1	3	6/7	3/7	7/7
	2	4		1			
Average number of larvae that responded out of total number active							
		3.8/6	1.4/3	2.3/7			
Percent number of larvae that responded of those active (%)							
		63	47	33			
Percent number of larvae active (%)							
		86	43	100			

Table A.6 Raw data for the number of control and neomycin treated 20 dph larvae eliciting a startle response to tapping two hours twenty minutes and two hours thirty minutes after treatment.

		Number of Larvae Responding to Tapping			**Number of Larvae Active and Upright Out of Total**		
	Tap #	Control	Stressed Control	5 mM	Control	Stressed Control	5mM
First Set: 2 h 20 min. after treatment							
	1	1	3	1	1/1	3/3	4/5
	2	1	2	2			
	3	1	2	0			
	4	1		1			
	5	1		1			
	6			0			
	7			0			
Second Set: 2 h 20 min. after treatment							
	1	1	1	1	1/1	3/3	4/5
	2	0	3	0			
	3	0	2	0			
	4	1	3	2			
	5			2			
	6			2			
Third Set: 2 h 20 min. after treatment							
	1	0	3	1	1/1	3/3	4/5
	2	1	1	1			
	3	0	2	0			
	4	1		1			
	5			0			
Average number of larvae that responded out of total number active across sets of tapping							
		0.69/1	2.2/3	0.83/4			
Percent number of larvae that responded of those active (%)							
		69	73.3	21			
Percent number of larvae active (%)							
		100	100	80			
First Set: 2 h 30 min. after treatment							
	1	1	3	0	1/1	3/3	4/5
	2	1	2	1			
	3	1	3	0			
	4			0			
Average number of larvae that responded out of total number active across sets of tapping							
		1/1	2.7/3	0.25/4			
Percent number of larvae that responded of those active (%)							
		100	90	6			
Percent number of larvae active (%)							
		100	100	80			

Table A.7 Mean prey capture rates per larva per hour (SE) by control and 5 mM neomycin treated larvae in either light (20 lx) or dark (0 lx) at 10, 13 , 17, and 20 dph. Means sharing the same letter are not significantly different at the 5% significance level. D=dark, C=control, L=light, N=neomycin

Age (dph)	D + C	D + N	L + C	L + N
10	[b]16.1 (0.25)	[a]6.1 (2.03)	[b]27.9 (0.68)	[a]7.6 (0.93)
13	[b]20.1 (0.60)	[a]5.4 (0.92)	[b]28.3 (0.44)	[b]24.8 (2.25)
17	[b]28.6 (0.65)	[a]5.7 (0.84)	[c]118.3 (1.29)	[c]97.1 (3.39)
20	[c]71.8 (5.86)	[a]4.9 (0.43)	[d]157.3 (2.96)	[d]140.7 (3.95)

Table A.8 Percent feeding incidence (SE) by control and 5 mM neomycin treated larvae in either light (20 lx) or dark (0 lx) at 10, 13 , 17, and 20 dph. Means sharing the same letter are not significantly different at the 5% significance level. D=dark, C=control, L=light, N=neomycin

Age (dph)	D + C	D + N	L + C	L + N
10	[bc]77.4 (7.8)	[e]19.1 (6.8)	[ab]85.7 (4.2)	[de]33.3 (12.8)
13	[ab]83.3 (6.4)	[de]35 (9.6)	[ab]83.6 (13.3)	[cd]48.4 (7.7)
17	[bc]63 (2.8)	[d]38 (7.7)	[b]72 (5.1)	[d]43 (17.7)
20	[cd]60 (5.8)	[d]37 (10.2)	[a]92 (3.0)	[c]61 (4.9)

Table A.9 Percent non-feeding incidence by larvae without inflated swimbladders in either the control or 5 mM neomycin treatment group fed in either light (20 lx) or dark (0 lx) at 10, 13 , 17, and 20 dph. n=percent non-feeders with no swimbladder/total non-feeders, D=dark, C=control, L=light, N=neomycin

Age (dph)	D + C	D + N	L + C	L + N	n D+C	n D+N	n L+C	n L+N
10	46	26	0	46	10/21	13/53	0/6	19/39
13	88	66	100	93	14/16	38/58	10/10	27/29
17	62	13	100	89	31/61	17/108	19/19	48/55
20	85	52	83	92	44/52	42/80	5/6	42/46

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