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The Distribution and Ontogeny of Neuromast Receptor Organs and a Comparison of Methods for Chemical Albation of the Lateral Line System in Two Cichlid Fishes

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THE DISTRIBUTION AND ONTOGENY OF NEUROMAST RECEPTOR ORGANS AND A COMPARISON OF METHODS FOR CHEMICAL ABLATION OF THE LATERAL LINE SYSTEM IN TWO CICHLID FISHES

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ABSTRACT

The lateral line system is composed of a series of mechanoreceptors called neuromasts, which are found on the head and trunk in specific patterns. On the head, larger canal neuromasts (CNs) are enclosed in cranial canals and smaller superficial neuromasts (SNs) are embedded in the skin in lines or clusters. Among species the lateral line canals can be narrow, widened, branched, or reduced. Through the use of fluorescent stains, Scanning Electron Microscopy, and cleared and stained specimens the distribution and ontogenetic appearance of SNs and CNs were mapped in two species of Lake Malawi cichlids with divergent adult lateral line morphologies: *Tramitichromis* sp. (narrow canals) and *A. stuartgranti stuartgranti* (widened canals).This study provides: 1) the first description of cranial neuromast distributions in representatives of the genera *Tramitichromis* and *A. stuartgranti* , 2) evidence that CN patterning is the same in the 2 taxa despite differences in adult canal morphology, 3) evidence that SN patterns (e.g., 9 groups of NM) are the same, but SN numbers vary between the two taxa, and 4) evidence that the timing and appearance of some SN groups varies between the two species.

Chemical and pharmacological ablation of neuromasts are methods frequently used to inactive the lateral line system. Fluorescent staining of neuromasts is also a common technique used to visually assess the effects of ablation on neuormasts. The two techniques, however, have only been used sequentially once before to verify that lateral line ablation occurred and that the behavior of the fish was a reflection of this.

The interpretation of the use of these methods and how they might differentially affect SNs and CNs is very ambiguous. This study provides the first detailed description of the ablation effects of Cobalt (II) chloride heptahydrate and Gentamicin on superficial and canal neuromasts using fluorescence staining (4-Di-2-ASP). Two species of Lake Malawi cichlids, *Tramitichromis* sp. and *A. stuartgranti stuartgranti*, were used in this study. Following treatment, it was determined that: 1) $CoCl_2$ in Ca^{++} free water and Gentamicin had comparable effects on SNs and CNs in both species, 2) Treatment with $CoCl_2$ in Ca^{++} free water and Gentamicin resulted in full recovery of both superficial and canal neuromasts by Day 4 or Day 7, 3) Treatment with $CoCl_2$ in tank water with Ca^{++} did not effectively ablate SNs and CNs on Day 0, when compared to $CoCl_2$ in Ca^{++} free water and Gentamicin, 4) Gentamicin does, in fact, affect SNs, which refutes previous experiments. The stain 4-Di-2-ASP proved to be a reliable and effective means of visually documenting the effects of Cobalt (II) chloride heptahydrate and Gentamicin on ablation and recovery of superficial and canal neuromasts in two cichlid species.

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PREFACE

This thesis was written and formatted following the guidelines presented by the University of Rhode Island Graduate School for Manuscript Form. There are two chapters: Distribution and Development of Cranial Neuromasts in two Cichlids with Divergent Adult Lateral Line Canal Morphology (Chapter 1), and Susceptibility and Recovery of Canal and Superficial Neuromasts to Cobalt Chloride and Gentamicin Ablation in Two Lake Malawi Cichlid Fishes: A Comparative Study (Chapter 2). This study was conducted under approved URI IACUC protocol # AN-08-11-005.

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INTRODUCTION

The mechanosensory lateral line system of fishes is composed of a series of receptor organs called neuromasts, which are found on the head and trunk. Two types of neuromasts are found in juvenile and adults of bony fishes: larger canal neuromasts enclosed in canals (canal neuromasts, CN), and smaller superficial neuromasts (SN) that are embedded in the skin in lines or clusters. SNs are sensitive to water flow velocity, and CNs are sensitive to water flow acceleration in response to flows outside of the canals (Coombs et al. 2001). CNs are located in lateral line canals located in a conserved subset of the dermal bones of the head. They are narrow, widened, branched, or reduced among teleosts (Webb, 1989b). Narrow canals, which are the most common, are well-ossified and are perforated by pores in precise locations. In contrast, widened canals, which have evolved convergently in a small number of teleost families (Webb, 1989a) have bony canal walls, but largely unossified canal roofs and the canals tend to be wider in diameter. The CNs sit beneath thin bony bridges and large bony pores are in the canal roof, which is covered by an epithelium that is pierced by very small pores that connect the fluid inside the canal with the external environment.

The analysis of neuromast migration and patterning and the genetic mechanisms underlying these processes have been studied extensively in zebrafish (Sapede et al., 2002; Ghysen and Dambly-Chaudiere, 2004), but the relationship of the distribution of neuromasts in embryos and young larvae to subsequent processes of neuromast maturation and canal morphogenesis has only been investigated in detail in

zebrafish (Webb and Shirey, 2003), and in a few cichlid species (Webb, 1989c; Tarby and Webb, 2003) all of which are characterized by narrow lateral line canals. The diversity found in the lateral line system of fishes has been attributed to differences in the pattern and timing of development (Webb, 1989a), but the development of the lateral line system in closely related species characterized by divergent adult morphologies is still lacking. Thus, information is needed on: 1) the distribution of CNs and SNs in juveniles and adults of narrow canal and widened canal species, and 2) the ontogeny of neuromast distribution and morphology in embryos and larvae of narrow and widened canal species. Two closely related cichlid species, *Tramitichromis* sp. (narrow canals) and *A. stuartgranti stuartgranti* (widened canals), were used in this study to represent these two lateral line canal morphologies.

Chemical and pharmacological ablations of neuromasts are methods frequently used to assess the impact of an inactive lateral line system on behavior. These techniques are commonly used on model species such as zebrafish and Mexican blind cave fish (Van Trump et al., 2010; Buck et al., 2012). Fluorescent staining of neuromasts is also a common technique used to visually assess the effects of ablation on neuromasts. The two techniques, however have only been used sequentially once before to verify that lateral line ablation occurred and that behavior was a reflection of this (Schwalbe et al., 2012).

Cobalt (II) chloride heptahydrate (Co^{2+}) has been shown to block the mechanoreceptor channels of hair cells in neuromasts of fish (Baumann & Roth, 1986; Karlsen and Sand, 1987), rendering the neuromast unresponsive to water flows. This effect was tested by Karlsen and Sand (1987) on the roach *(Rutilus rutilus).* They

found the effects of cobalt were reversed (behavioral reaction to a vibrating ball) after 2-3 weeks, through an increase in calcium (i.e., placing them in normal tank water).

Aminoglycoside antibiotics selectively block transduction channels including Ca^{++} channels (Hudspeth and Kroese, 1983; Kroese et al., 1989; Pichler et al. 1996) in sensory hair cells. As in the inner ear, neuromast hair cells are mechanoreceptors in which the opening of Ca^{++} channels is essential for their function. The effects of aminoglycosides on lateral line function have been assessed visually and behaviorally (Montgomery et al., 1997; Coombs et al., 2001). The effects of gentamicin on superficial and canal neuromasts in the Mexican blind cavefish (*Astyanax mexicanus*) and zebrafish (*Danio rerio*) have been investigated, with the finding that both types of neuromasts are inactivated (Van Trump et. al. 2010).

Hair cells can be visualized *in vivo* using fluorescent styryl dyes, like DASPEI (2-(4-(dimethylamino)styryl)-N-ethylpyridinium iodide) , FM1-43 ((n-(3triethylammoniumpropyl)-4-(4-(dibutylamino)styryl) pyridinium dibromide), and 4-Di-2-ASP. The fluorescent stain 4-Di-2-ASP enters the mechanotransduction Ca^{++} channels of hair cells and stains mitochondria (Magrassi et al., 1987), allowing visualization of functional hair cells and support cells in superficial and canal neuromasts.

Most ablation and staining research has been conducted on fish with narrow lateral line canals, either zebrafish (Chiu et al., 2008; Coffin et al., 2009; Harris et al., 2003; Ou et al., 2007; Owens et al., 2007; Van Trump et al., 2010; Buck et al., 2012) or Mexican blind cave fish (Van Trump et al., 2010; Buck et al., 2012). The two

species used in this study represent narrow (*Tramitichromis*) and widened (*Aulonocara*) canal morphologies.

The interpretation of the use of these methods and how they might differentially affect SNs and CNs is very ambiguous. Thus, a clear description of which staining techniques and ablation methods work best on narrow and widened canal species is needed in order to verify previous lateral line ablation behavior experiments.

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Distribution and Development of Cranial Neuromasts in Two Cichlids with Divergent Adult Lateral Line Canal Morphology

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ABSTRACT

The lateral line system is composed of a series of mechanoreceptors called neuromasts, which are found on the head and trunk in specific patterns. On the head, larger canal neuromasts (CNs) are enclosed in cranial canals and smaller superficial neuromasts (SNs) are embedded in the skin in lines or clusters. Among species the lateral line canals can be narrow, widened, branched, or reduced. Through the use of fluorescent stains, Scanning Electron Microscopy, and cleared and stained specimens the distribution and ontogenetic appearance of SNs and CNs were mapped in two species of Lake Malawi cichlids with divergent adult lateral line morphologies: *Tramitichromis* sp. (narrow canals) and *A. stuartgranti stuartgranti* (widened canals).This study provides: 1) the first description of cranial neuromast distributions in representatives of the genera *Tramitichromis* and *A. stuartgranti* , 2) evidence that CN patterning is the same in the 2 taxa despite differences in adult canal morphology, 3) evidence that SN patterns (e.g., 9 groups of NM) are the same, but SN numbers vary between the two taxa, and 4) evidence that the timing and appearance of some SN groups varies between the two species.

INTRODUCTION

The mechanosensory lateral line system of fishes is composed of a series of receptor organs called neuromasts, which are found on the head and trunk. Two types of neuromasts are found in juveniles and adults of bony fishes: larger canal neuromasts enclosed in canals (canal neuromasts, CN), and smaller superficial neuromasts (SN) that are embedded in the surface of the skin in lines or clusters. Neuromasts detect water flows. Superficial neuromasts are sensitive to flow velocity, and canal neuromasts are sensitive to accelerations in response to flows outside of the canals (Coombs et al. 2001). Cranial lateral line canals are located in a conserved subset of the dermal bones of the head. They are narrow, widened, branched, or reduced among species (Webb, 1989b). Narrow canals, which are most common among fishes, are well-ossified and are perforated by pores in precise locations. In contrast, widened canals, which have evolved convergently in a small number of fish families (Webb, 1989a) have bony canal walls, but largely unossified canal roofs and the canals tend to be wider in diameter. The CNs sit beneath thin bony bridges and large bony pores are in the canal roof, which is covered by an epithelium that is pierced by very small pores that connect the fluid inside the canal with the external environment (see Fig. 1 for size comparison).

Neuromasts in narrow canals tend to be oval in shape, with their major axis and an elongated sensory strip (containing the sensory hair cells) parallel to the long axis of the canal. Hair cells in the sensory strip are surrounded by non-sensory support cells that secrete the cupula and determine the overall shape of the neuromast (Webb,

2000a). In widened canals, neuromasts are generally much larger than those in narrow canals and have a prominent axis perpendicular to the canal axis, but the orientation of the hair cells is parallel to the canal axis. As in neuromasts in narrow canals, support cells surround the sensory strip so that neuromasts in widened canals often have a diamond shape (e.g., Jakubowski, 1967).

The formation of the lateral line system begins during embryogenesis and has been described as three phases (Webb 1989b). First, the primordium of the neuromasts, which is derived from cranial lateral line placodes, migrates from the head to the tail and establishes the distribution of neuromasts along the body (Lopez-Schier et al. 2004). In addition to establishing the distribution of neuromasts, each lateral line placode gives rise to the sensory neurons to innervate each neuromast (Münz, 1979; Gibbs 2004). The second phase involves the maturation and growth of neuromasts in length and width (Tarby and Webb 2003, Webb and Shirey 2003). In the third phase, the lateral line canals form in four stages in which individual neuromasts gradually become enclosed in tubular canal segments (Tarby and Webb, 2003; Webb and Shirey 2003) and the canals form and become integrated within the dermal cranial bones.

The analysis of neuromast migration and patterning and the genetic mechanisms underlying these processes have been studied extensively in zebrafish (Sapede et al., 2002; Ghysen and Dambly-Chaudiere, 2004), but the relationship of the distribution of neuromasts in embryos and young larvae to subsequent processes of neuromast maturation and canal morphology has only been investigated in detail in

zebrafish (Webb and Shirey, 2003) and in a few cichlid species (Webb, 1989c; Tarby and Webb, 2003) all of which are characterized by narrow lateral line canals.

The diversity found in the lateral line system of fishes has been attributed to differences in the pattern and timing of development (Webb, 1989a), but the development of the lateral line system in closely related species characterized by divergent adult morphologies is still lacking. This study addresses a fundamental question: what is the relationship between neuromast patterning and adult lateral line morphology? It will test the hypothesis that two related species with divergent adult lateral line morphologies (narrow and widened cranial lateral line canals) have the same complement and number of canal and superficial neuromasts. Thus, the approach of this project was to: 1) describe the distribution of canal and superficial neuromasts in juveniles and adults of two Lake Malawi cichlid species: one with narrow canals (*Tramitichromis* sp.) and one with widened canals (*A. stuartgranti stuartgranti*), and 2) describe the ontogeny of neuromast distribution and morphology in embryos and larvae of these two species.

MATERIAL AND METHODS

Study Species

Tramitichromis sp. and *A. stuartgranti stuartgranti* are mouth-brooding cichlids endemic to Lake Malawi (Africa), which are commercially available and easily reared in a lab setting. Breeding tanks in two flow- through systems were lined with a mixture of sand and gravel, provided with mechanical and biological filtration and kept at 80 ±1°F and salinity of 1±0.5 ppt with a 12L:12D hour light regime. A

breeding group of one male and several females were provided with PVC pipe and rocks to mimic their natural habitat in order to promote breeding. Animals were fed daily on a varied diet (protein pellets, live brine shrimp, a pea/shrimp mixture, or an algae/yolk/earthworm protein flake mixture). The date on which a brood was noticed (e.g., expanded buccal cavity observed in female) was recorded, an indication that fertilization had occurred within 24 hours. Broods of yolk sac larvae (at least 4 days post fertilization (dpf)) were removed from the mouth of brooding females and raised in round-bottomed glass flasks with slow water exchange within 1 liter tanks in an Aquatic Habitats recirculating rack system. After yolk sac absorption, actively swimming larvae would swim out of the flasks, and were then fed a flake mixture of egg yolk, earthworm protein, and algae. Ontogenetic series were generated from 6 broods (all half siblings; same father) of *Tramitichromis* sp. and 7 broods (relationships unknown) of *A. stuartgranti* by sampling fish every 1-3 days (e.g., 1- 2 mm SL intervals).

Neuromast Staining and Imaging

The fluorescent mitochondrial stains 4-Di-2-ASP (4-(4-(diethylamino) styryl)-N-methylpyridinium Iodide; Sigma Aldrich) and DASPEI (2-(4-(dimethylamino) styryl)-N-ethylpyridinium iodide; Sigma Aldrich) were used to document the spatial distribution of neuromasts on the head in embryos, larvae and adults. Neuromasts in the two species were visualized using 4-Di-2-ASP (>15 dpf) or DASPEI (4-15 dpf, to avoid interference with strong fluorescence by the gills caused by 4-Di-2-ASP).

Live fish were immersed for 5 minutes in a 0.0024% (63 µM) solution of 4-Di-2-ASP in tank water or for 30 minutes in a 0.01% solution of DASPEI in tank water and were then anaesthetized in 0.33% buffered MS-222 (Tricaine methanesulfonate; Sigma Aldrich) till movements ceased. Light and fluorescent images of the lateral, dorsal and ventral surfaces of the head of each fish were obtained at 2x magnification using a Nikon dissecting microscope scope (Model SMZ1500) using a Spot digital camera (Model 25.22 Mp Color Mosaic) and Spot software (v. 5.0; Diagnostic Instruments, Sterling Heights, MI). Tracings were made from fluorescent digital images in ventral, lateral, and dorsal views to detail the distribution of canal and superficial neuromasts.

Six adult *A. stuartgranti* (>154 dpf; 16-26 mm SL) and five adult *Tramitichromis* sp. (>99 dpf; 15-21 mm SL) were used to determine canal and superficial neuromast distributions in adults (see Fig. 4). In addition, a total of 40 larval and juvenile *A. stuartgranti* (~5-154 dpf; 6-28 mm SL) and 61 larval and juvenile *Tramitichromis* sp. (~4-99dpf; 4-18 mm SL) were used to describe the ontogeny of neuromast distributions.

Cleared and Stained Material

Tramitichromis sp. and *A. stuartgranti* previously cleared and stained (for bone and cartilage; Potthoff, 1984) were used to identify the location of lateral line canals within the dermal bones of the head. Fluorescent images (generated as above) could then be superimposed over images of cleared and stained fish to identify the precise

location of canal neuromasts within the canals. Superficial neuromasts were assigned names based on Peters (1973) description of neuromast distributions in tilapia.

Scanning Electron Microscopy

SEM (done by J.F. Webb) was used to reveal neuromast size, shape and hair cell orientation. Larval and juvenile *Tramitichromis* sp.(11-13 mm SL, n=12) and *A. stuartgranti* (11.5 – 16 mm SL, n=24) that had been fixed in 10% formalin in Phosphate Buffered Saline for up to several months (including those used for fluorescent imaging) were dehydrated in ethanol (50%-2 hours; 70% overnight, 80%, 95%, 100%x3, each for ~1 hour). Fish were critical-point-dried out of liquid CO_2 (Tousimis Samdri-780A) coated with gold alloy (Leica MED 020), and mounted on aluminum stubs with adhesive carbon discs. Specimens were viewed with a Zeiss NTS Supra 40VP scanning electron microscope at 3 KV at the Marine Biological Laboratory, Woods Hole, MA.

RESULTS

Tramitichromis sp. and *A. stuartgrant* , both mouth brooders, hatch at about 4-5 mm total length (TL) at ~5 days post-fertilization (dpf). Their prominent yolk sac is absorbed by about 20 dpf, just before being released from the mother's mouth (by ~21 dpf) at which point the fish are juveniles (Fig.2).

A small number of neuromasts are present on the skin at hatch in both species. Presumptive CNs as well as other SNs (which will remain on the skin) are easily distinguished by size at an early age (~6 dpf in *Tramitichromis* sp.; ~7 dpf in *A.*

stuartgranti), well before canals begin to develop. In older larvae and early juveniles, the presumptive CNs increase in size and SEM revealed their diamond shape. SNs are smaller than CNs and are either round (e.g., in the clusters on the mandible) or diamond -shaped with an elongate sensory strip (e.g., on the cheek area). Hair cell orientation in presumptive CNs is always parallel to the axis of the canal, whereas hair cell orientation in SNs varies among groups or series, and may be parallel or perpendicular to the body axis.

Distribution and Morphology of Canal Neuromasts (CNs)

The number and distribution of CNs is the same in both species. Five supraorbital canal neuromasts (SO1-5) are contained in the supraorbital (SO) canal and are located dorsal to and medial to the orbits (Fig. 3 A and D; Fig. 4 A and D). One canal pore is found between the positions of adjacent canal neuromasts along the canal. Neuromast SO1 is located in the tubular nasal bone, while neuromasts SO2–SO5 are located in the portion of the SO canal that runs along the frontal bone. The canal begins rostral to the naris with a terminal pore that is present in both species. In *Tramitichromis* sp., SO1 is medial to the olfactory organ and is not easily visualized in fluorescent images due to the strong staining of the olfactory epithelium (Fig. 3 B and E), so histological material (N. Bird, pers. comm.) was used to identify this neuromast. In *A. stuartgranti*, SO1 is positioned further towards the dorsal midline and was easily visualized. A common median pore connecting the left and right SO canals is found between the positions of SO3 and SO4. The terminal pore of the SO canal is caudal to

SO5 and connects where the SO and infraorbital (IO) meet (canals not indicated in figure; Fig.2 A and D; Fig. 3 A and D).

The mandibular (MD) canal contains five neuromasts (MD1-5; Fig. 3 C and F; Fig. 4 C and F). MD1 through MD4 are enclosed in the portion of the canal in the dentary bone. MD5 is contained in the short canal segment in the anguloarticular bone. The anterior end of the canal begins with a terminal pore, followed by MD1. One pore is found between adjacent neuromasts along the canal. The posterior terminal pore of the MD canal in the dentary bone opens into the anguloarticular bone just after MD4; MD5 is contained within the canal segment in the anguloarticular. The terminal pore of the MD canal is found at the junction between the MD canal and the preopercular (PO) canal (Fig. 3 C and F; Fig. 4 C and F).

The infraorbital (IO) canal contains nine neuromasts (IO1-9) and follows the circumference of the orbit, beginning in the lacrimal bone and then continuing into the tubular infraorbital ossicles (Fig. 3 B and E; Fig. 4 B and E). This series begins below the naris and IO1-3 are found in the lacrimal bone; the other six IO neuromasts (IO 4-9) are found in the tubular infraorbital ossicles. A bony pore is found at a position between each two neuromasts in the series. The IO canal terminates in a pore, which is also the terminal pore for the SO series (Fig. 3 B and E).

The L-shaped preopercular (PO) canal, which contains six neuromasts (PO1-6), connects rostrally with the terminal pore of the mandibular canal and continues dorsally, terminating caudally to the orbit (Fig. 3 B and E).

Distribution and Morphology of Superficial Neuromasts

Superficial neuromasts were found in nine series in both *Tramitichromis* sp. and *A. stuartgranti* and were named using a scheme for *Tilapia* (Peters, 1973; Table 1). Both species have the same complement of SN series, but the number of neuromasts in each series differs. The nine SN series can be organized into dorsal, lateral and ventral series (e.g., See Table 1).

The dorsolateral (DL) series consists of 7-9 neuromasts (Table 1) in both species, and forms a curved line on the top of the head at a 45-degree angle to the body axis (Fig. 3 A and D; Fig. 4 A and D).

The circumnasal series (ZN) is lateral or ventral to the naris and is composed of 8-10 neuromasts in *Tramitichromis* sp.and 7-10 neuromasts in *A. stuartgranti* (Fig. 3 B and E; Fig. 4 B and E).

The supranasal series (SUN) is found on the dorsal surface of the "snout", and consists of 3 transverse lines of SNs (SUN1-3) in *Tramitichromis* sp. and 4 transverse lines (SUN1-4) in *A. stuartgranti*. The left and right portions of these lines, which are presumably innervated by different nerves, cannot be clearly distinguished. Adult *Tramitichromis* have a total of 10-12 SUN neuromasts (left + right), and *A. stuartgranti* have a total of 21- 28 SUN neuromasts (left + right; Fig. 3 A and D; Fig. 4 A and D).

The supratemporal cross series (STC) is perpendicular to the body axis in a transverse line caudal to the eyes on the dorsal side of the head. It is comprised of 6-10 neuromasts in both *A. stuartgranti* and *Tramitichromis* sp.(Fig. 3 A and D; Fig. 4 A and D).

The supracleithral cross series (ScC) is an irregular transverse line of SNs just rostral to the dorsal fin and caudal to the STC series in both species (Fig. 3 A and D; Fig. 4 A and D). Like the SUN series, the neuromasts of the right and left sides cannot be clearly distinguished. *Tramitichromis* sp. has a total of 6-9 neuromasts in this series (left + right), while *A. stuartgranti* tends to have a higher number with a total of 9-12 neuromasts.

The frontal cross series (FC) runs vertically and is composed of 1-4 neuromasts in *Tramitichromis* sp. and 5-7 neuromasts in *A. stuartgranti* (Fig. 3 B and E; Fig. 4 B and E). It extends from the caudal end of the DL series and is visible in the dorsal view.

The opercular vertical series (OVS) runs dorso-ventrally and is caudal to the PO canal series; it extends from the level of canal neuromast PO3 dorsally to the level of canal neuromast PO6. There was a difference in the quantity of neuromasts in this series between the two species. The OVS neuromasts in *Tramitichromis* sp. are in a linear series, but it appears that neuromast proliferation caudal to the line of neuromasts has resulted in the formation of a small neuromast cluster at the dorsal and ventral ends of the series, resulting in a total of 13-20 neuromasts (Fig. 3 B and E; Fig. 4 B and E). *A. stuartgranti* shows a similar pattern, but the total number of neuromasts in this series was 16-26. In both species, SEM reveals that the sensory strip and the physiological orientation of the hair cells of the OVS neuromasts is rostro-caudal, thus perpendicular to the line of neuromasts in this series (Fig. 3 B and E; Fig. 4 B and E).

The cheek vertical series (CVS) runs vertically between the IO canal and PO canal neuromast series (Fig. 3 B and E; Fig. 4 B and E). Its ventral-most neuromast is

positioned just ventral to canal neuromasts PO2 and PO3 and runs in a linear series, dorsally to a position between canal neuromasts IO8 and PO6. In both species, the CVS series consists of 8-12 neuromasts (Fig. 3 B and E; Fig. 4 B and E). In contrast to the OVS series, the sensory strip and the physiological orientation of the hair cells of the CVS neuromasts is dorso-ventral, thus parallel to the line of neuromasts in this series.

The superficial mandibular (SM) series consist of four spatially distinct clusters (SM1-4) in *Tramitichromis* sp. and *A. stuartgranti.* The clusters are positioned between the bony pores (which are quite large in *Aulonocara*; Fig. 5) of the mandibular canal. The number of SNs within each cluster increases with fish size in both species, with neuromast numbers reaching 14-18 neuromasts in the series (Fig. 3 C and F; Fig. 4 C and F). In both species, these neuromasts are round and extremely small (10-20 µm diameter; Fig. 5 F), and the orientation of the hair cells is perpendicular to the axis of the MD canal.

Ontogeny of Canal Neuromast Distributions

The number of presumptive CNs increases in late stage embryos and early larvae and stabilizes in larvae of 7-8 mm SL (~10-11 dpf), prior to the initiation of canal development (Fig. 6). *Tramitichromis* sp. CNs tend to be smaller than those in *A. stuartgranti*, as revealed by fluorescent images (hair cell population in sensory strip) and SEM (entire neuromast). For example, sensory strip length of MD CN for *Tramitichromis* sp. are ~20µm and ~30µm for *A. stuartgranti* in juveniles (~11 mm SL; Fig. 5).

The first presumptive SO canal neuromast is visible on the epithelium in *Tramitichromis* sp. around 4 dpf (4 mm SL). By 10 dpf (8 mm SL), *Tramitichromis* sp. have a full complement of SO NMs (Fig. 6; Table 1). In contrast, the first presumptive SO canal neuromasts appear at 5 dpf (5 mm SL) in *A. stuartgranti.* By 8 dpf (6 mm SL), all 5 SO neuromasts are present and by 18 dpf, all SO canals appear to be enclosed. *Tramitichromis* sp. acquires a full set of SO canal neuromasts in a growth interval from 4- 8 mm SL, while *A. stuartgranti* obtains a full set in a much smaller growth interval of 5-6 mm SL).

The MD canal neuromasts first appears at 5 dpf (4 mm SL) in *Tramitichromis* sp., with completion of the series at about 7 dpf (8 mm SL) (Fig. 6; Table 1). Mandibular canals appear to be fully enclosed about one week later at 15 dpf. In *A. stuartgranti*, the first mandibular CN appears at 4 dpf (5 mm SL), just before hatch (Table 1). A full complement of NM in the MD series (total 5 NM) is present at 6 dpf (6 mm SL), and the canals appear to be completely closed by about 16 dpf. It is the first set of canal neuromasts to form on *A. stuartgranti*.

In *Tramitichromis* sp., the first presumptive IO neuromast appears at 4 dpf and by 7 dpf, the series is complete (Fig. 6; Table 1). In *A. stuartgranti* the IO series first appears at 5 dpf with the complete set (9 NM) of neuromasts visible by ~11 dpf (Fig. 6; Table 1). This canal appears to be enclosed at about 40 dpf, indicated by pigmentation in the skin obscuring a full view of the canal neuromasts that lie beneath the canal.

In *Tramitichromis* sp., the first presumptive PO canal neuromast appears at 4 dpf and is complete around 7 dpf (Fig. 6; Table 1). In *A. stuartgranti* , the first PO

neuromast is visible on the epithelium at 6 dpf. By 8 dpf, all 6 canal neuromasts are formed and by 17 dpf, the canal appears to be fully enclosed (Fig. 6; Table 1).

Ontogeny of Superficial Neuromast Distributions

In contrast to the canal neuromasts whose number stabilizes rather early, the number of SNs continues to increase through the larval and juvenile stages, with variation in the rate of neuromast addition between species and among SN series within a species (Fig. 7). The first appearance of SNs in the different series occurs from 4-15 dpf in *Tramitichromis* sp. and 4-11 dpf in *A. stuartgranti* , which is either during or after the first appearance of the CNs (at 4-5 dpf in *Tramitichromis* sp., 4-6 dpf in *A. stuartgranti* ; Table 1) and after the age at which the final number of CNs is reached (9-10 dpf; 7-8 mm SL). By 5 dpf (just after hatch), CNs are noticeably larger than SNs in both species (e.g., Fig. 5).

The timing of the first appearance of SN series varies with position on the head (Table 1; Fig. 8). For instance, in *Tramitichromis* sp., neuromasts of the SUN, CVS and SM series, between the nares (on the dorsal surface of the "snout"), laterally on the cheek and ventrally on the mandible, respectively, all first appear at 4 dpf (before hatch). Then neuromasts of the DL and ZF series and the STC and ScC series (all dorsal) appear at 6-7 dpf. Finally, the OVS series on the cheek and the FC series, dorsal and caudal to the orbit first appear at ~8 dpf and a week later, at 15 dpf. In contrast, in *A. stuartgranti* , neuromasts of the SM series (SM1) on the mandible appear at 4 dpf (before hatch), but other series, the dorsal SUN, STC series, and lateral

CVS series appear three days later, at 7 dpf. Neuromasts of the dorsal DL and ZN and lateral OVS series all appear about a day later, at 8 dpf. Finally neuromasts in the dorsal ScC and lateral FC series, appear several days later, at 11 dpf. Three of the nine SN series (STC, OVS, SM) first appear on the same day in both species. Five of the nine SN series (DL, ZN, SUN, ScC, CVS) start to appear earlier in *Tramitichromis* sp. than in *A. stuartgranti*, but the initial appearance of the last SN series to form in both species starts 4 days earlier in *A. stuartgranti* (ScC, FC) than in *Tramitichromis* sp. (FC; Table 1).

In both species, the number of SNs in each series increases with fish size and the total number of SNs on the head continues to increase throughout the juvenile period (Fig. 7). The rate of neuromast addition appears to vary among series. For instance, neuromasts of the STC series first appears at 7 dpf in both species, but *Tramitichromis* sp. and *A. stuartgranti* juveniles of comparable sizes, have 3-5 SN's in this series (Table 1). In contrast, neuromasts of the OVS series first appears at 8 dpf in both species, but juvenile *Tramitichromis* sp. and *A. stuartgranti* have 13-20 and 16-26 SNs respectively indicating that the rate of addition is likely different in the two species. Finally, neuromasts of the SUN series (left and right) first appears at 4 dpf in *Tramitichromis* sp. and 7 dpf in *A. stuartgranti* , but their juveniles have 10-12 SNs and 21-28 SNs respectively, indicating that the rate of addition in *A. stuartgranti* must be higher than in *Tramitichromis* sp.

The overall pattern of SN proliferation appears to be consistent in *Tramitichromis* sp. and *A. stuartgranti* , but varies somewhat among SN series (Fig. 8). The first two ZN neuromasts appear immediately rostral and caudal to the naris,

then SNs are added further away from the naris. The STC and ScC neuromasts first appear laterally and proliferate medially. The first SNs of the CVS and OVS series appear ventrally and proliferate dorsally. The symphyseal SM neuromasts (SM1) appear quite early and do not proliferate, while one neuormast appears in the position of each of the three other SM clusters and neuromast proliferation occurs within each cluster.

DISCUSSION

The present study expands our knowledge of the biology of Lake Malawi cichlids and the morphology and development of the lateral line system in *A. stuartgranti stuartgranti* and *Tramitichromis sp*. Fluorescent images, cleared and stained specimens, and SEMs provided us with extensive information regarding canal morphology, neuromast ontogeny and distribution, and hair cell orientation.

This study provides: 1) the first description of cranial neuromast distributions in *Tramitichromis* and *A. stuartgranti* , 2) shows that CN patterning is the same despite differences in adult canal morphology, 3) shows that SN patterns (e.g., 9 groups of NM) are the same, but SN numbers vary between the two species, and 4) shows that the timing and appearance of some SN groups varies between species. The two species investigated in this study both possess lateral line characteristics that are indicative of teleosts more broadly (Webb, 1989b) including: 1) four major cranial lateral line canals, 2) size differences between CNs and SNs, and 3) the organization of SNs into distinct clusters and lines.

Canal and superficial neuromasts in early larval zebrafish have previously been mapped using fluorescent markers and have been named according to both their location and innervation (Raible and Kruse, 2000). When we labeled CNs and SNs with 4-Di-2-ASP and compared them with other cichlid species with narrow canals (i.e., *Oreochromis spp*., Peters, 1973; *Archocentrus nigrofasciatus*, Tarby and Webb, 2003), we found that CN distribution and number is identical. This was not surprising, as subsequent differences in CN maturation (resulting in differences in CN size between species) and the degree of ossification in the canal roof result in divergent lateral line morphology, not the differences in initial CN number or distribution. Superficial neuromasts in both species are similar in their distribution, but the differences were found in the number of neuromasts. Most SNs are closely associated with CN series and are not randomly distributed over the head (Fig. 3 and Fig. 4), as in other teleost fishes (reviewed in Coombs et al. 1988). In teleosts, SNs also typically show varying physiological orientations (due to polarization of hair cells within NMs) depending on what SN group they are found in (Janssen et al., 1987; Song and Northcutt, 1991; Coombs and Montgomery, 1994). Based on SEMs, it was determined that this is also the case for the SNs in *Tramitichromis* sp. and *A. stuartgranti* (Fig. 5). The SNs found in specific groups, lines, or clusters, are assumed to be innervated by the same branch of a lateral line nerve, although more work needs to be done to verify this in the two study species. The greatest difference in lateral line morphology between *Tramitichromis* sp. and *A. stuartgranti* (besides canal morphology) is not found in the distribution of CNs, but in the proliferation of SNs within specific series.

Neuromast number and distribution is important in understanding the evolutionary relationships among fish taxa (Nakae et al., 2011;Nelson 1969; Nakae and Sasaki 2010). The data presented here points to differences in adult lateral line canal morphology (narrow vs. widened canals) that are correlated with differences in CN maturation (resulting in differences in CN size between species), and the degree of lateral line canal morphogenesis (degree of ossification of canal roof). They do not suggest that differences in CN number or distribution are related to the evolution of widened canals from narrow canals. In order to fully understand the evolution of the four lateral line canal morphologies (narrow, widened, branched, and reduced), additional specimen from a range of taxa still need to be examined.

Table 1.1. Timing of appearance of canal and superficial neuromasts in larvae and juveniles and number of neuromasts in older juveniles of *Tramitichromis(TRA)* and *A. stuartgranti (AU).* Data from left side only-excluding SUN and ScC which are bilateral count.

Neuromast Series	**Age when NM first appear**		**# NM in older juveniles**	
	TRA	***AU***	***TRA***	***AU***
Canal Neuromasts				
Supraorbital (SO)	4 dpf	5 dpf	5	5
Mandibular (MD)	5 dpf	4 dpf	5	5
Infraorbital (IO)	4 dpf	5 dpf	9	9
Preopercular (PO)	4 dpf	6 dpf	6	6
Superficial Neuromasts				
Dorsal				
Dorso-Lateral (DL)	6 dpf	8 dpf	7-9	7-9
Circumnasal (ZN)	6 dpf	8 dpf	8-10	7-10
Supranasal (SUN; multiple lines)	4 dpf	7 dpf	10-12	21-28
Supratemporal Cross (STC)	7 dpf	7 dpf	3-5	3-5
Supracleithrum Cross (ScC)	7 dpf	11 dpf	6-9	9-12
Lateral				
Frontal Cross (FC)	15 dpf	11 dpf	1-4	5-7
Opercular Vertical Series (OVS)	8 dpf	8 dpf	13-20	16-26
Cheek Vertical Series (CVS)	4 dpf	7 dpf	8-12	8-12
Ventral				
Superficial Mandibular (SM)	4 dpf	4 dpf	9-14	16-18

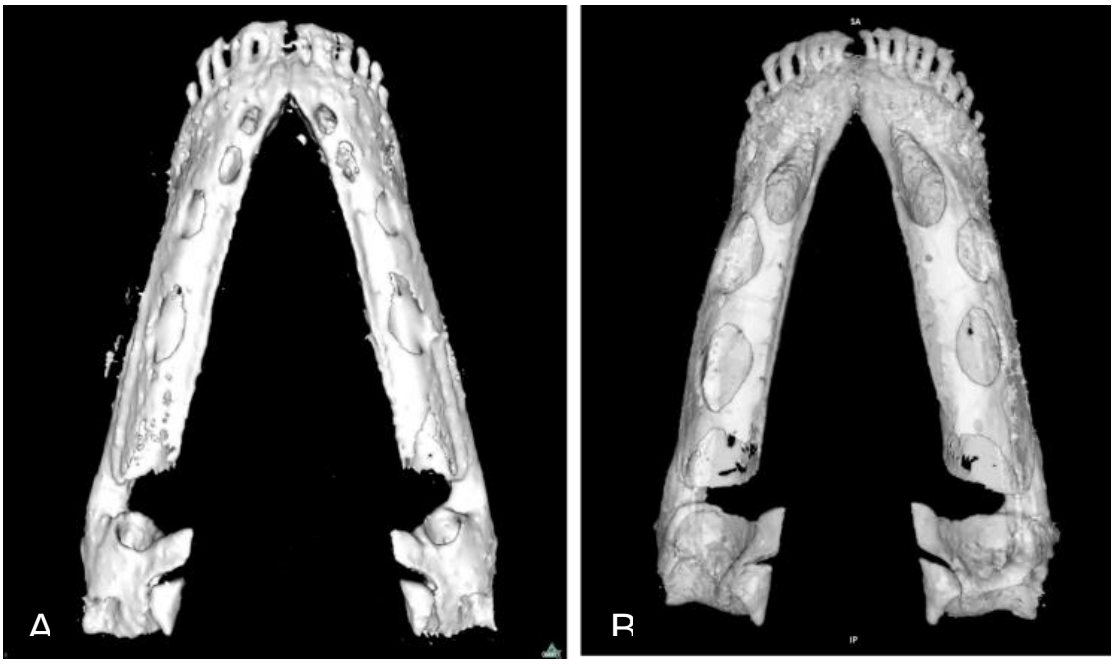

Fig.1.1 .Three-dimensional reconstructions of the mandible (ventral view) from CT scans (16 µm slice thickness) of A) *Tramitichromis sp.* (narrow canals) and B) *A. stuartgranti stuartgranti* (widened canals). Note significantly larger canal pores in *Aulonocara*.

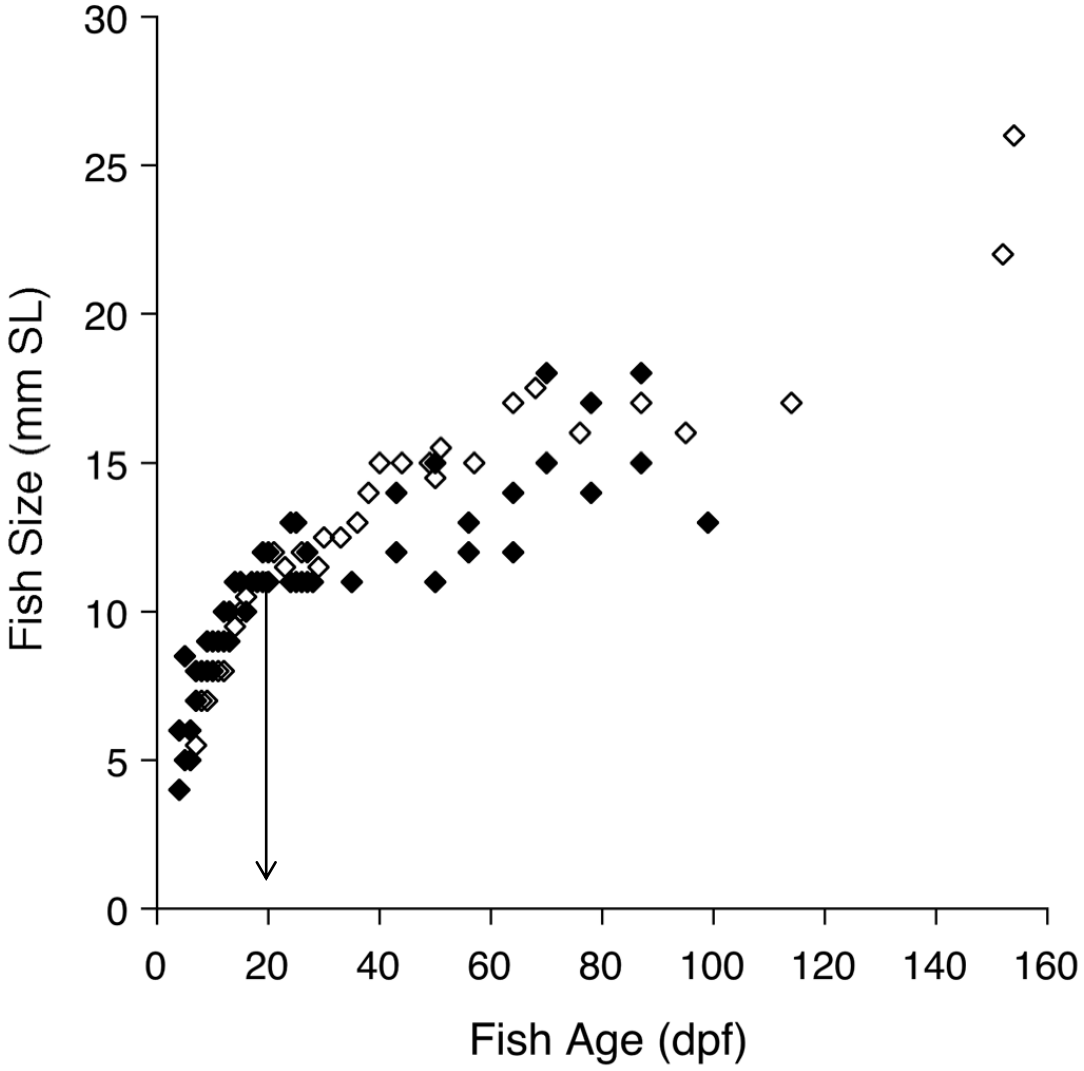

Fig.1.2 .Growth of *Tramitichromis* sp. (black diamonds; n=61) and *A. stuartgranti* (white diamonds; n=41) used in this study (ontogeny). Arrow indicates age at which juveniles typically leave the mother's mouth.

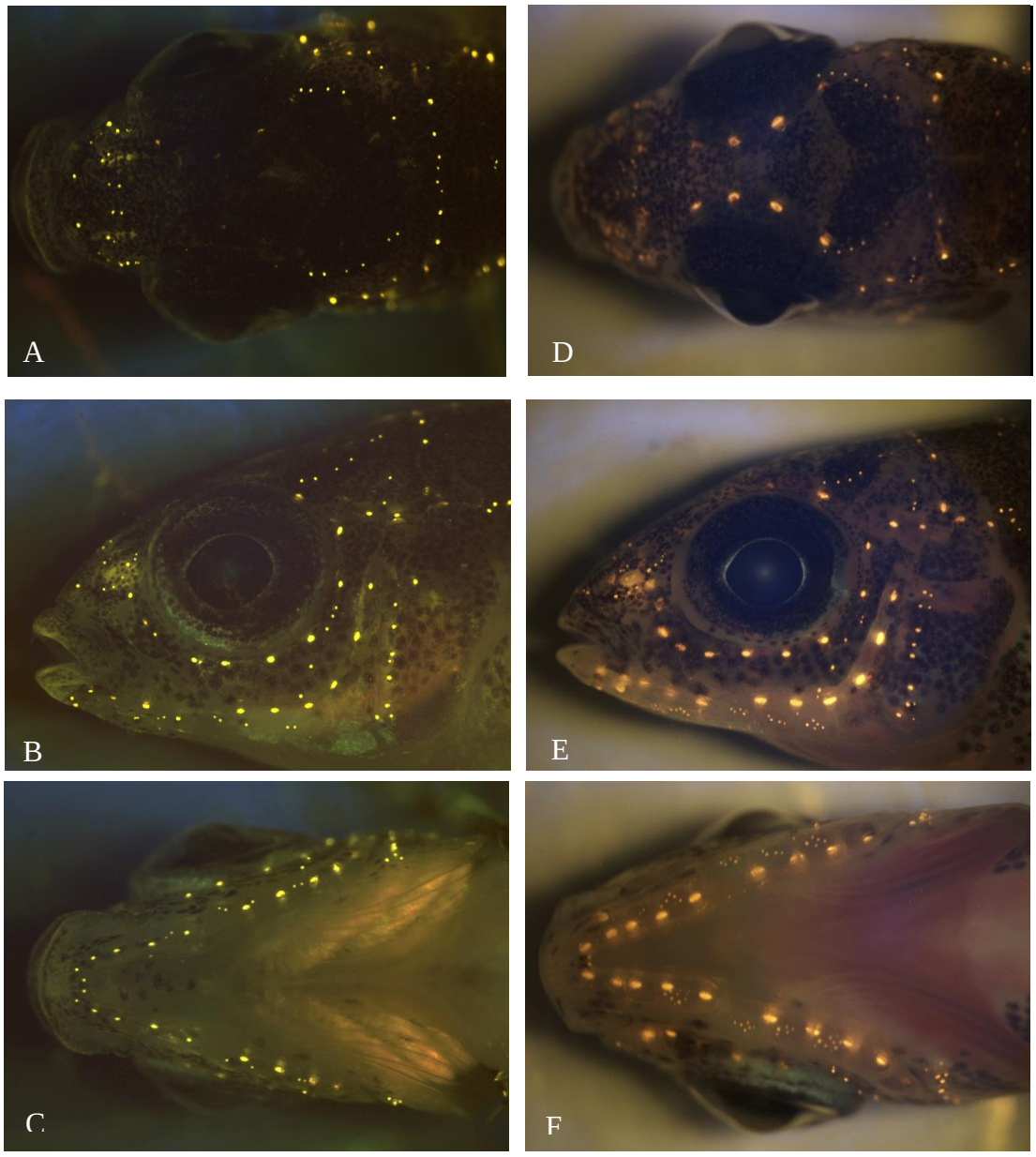

Fig. 1.3 . Canal (CN) and superficial (SN) neuromast distributions in dorsal, lateral and ventral fluorescent images. Fluorescent images of *Tramitichromis* sp. (A-C; narrow canals; 18 mm SL) and *A. stuartgranti* (D-F; widened canals, 21 mm SL). Sensory strip (hair cells) of CNs and SNs give a positive fluorescent signal. Note that CNs in *A. stuartgranti* are larger than those in *Tramitichromis* sp.

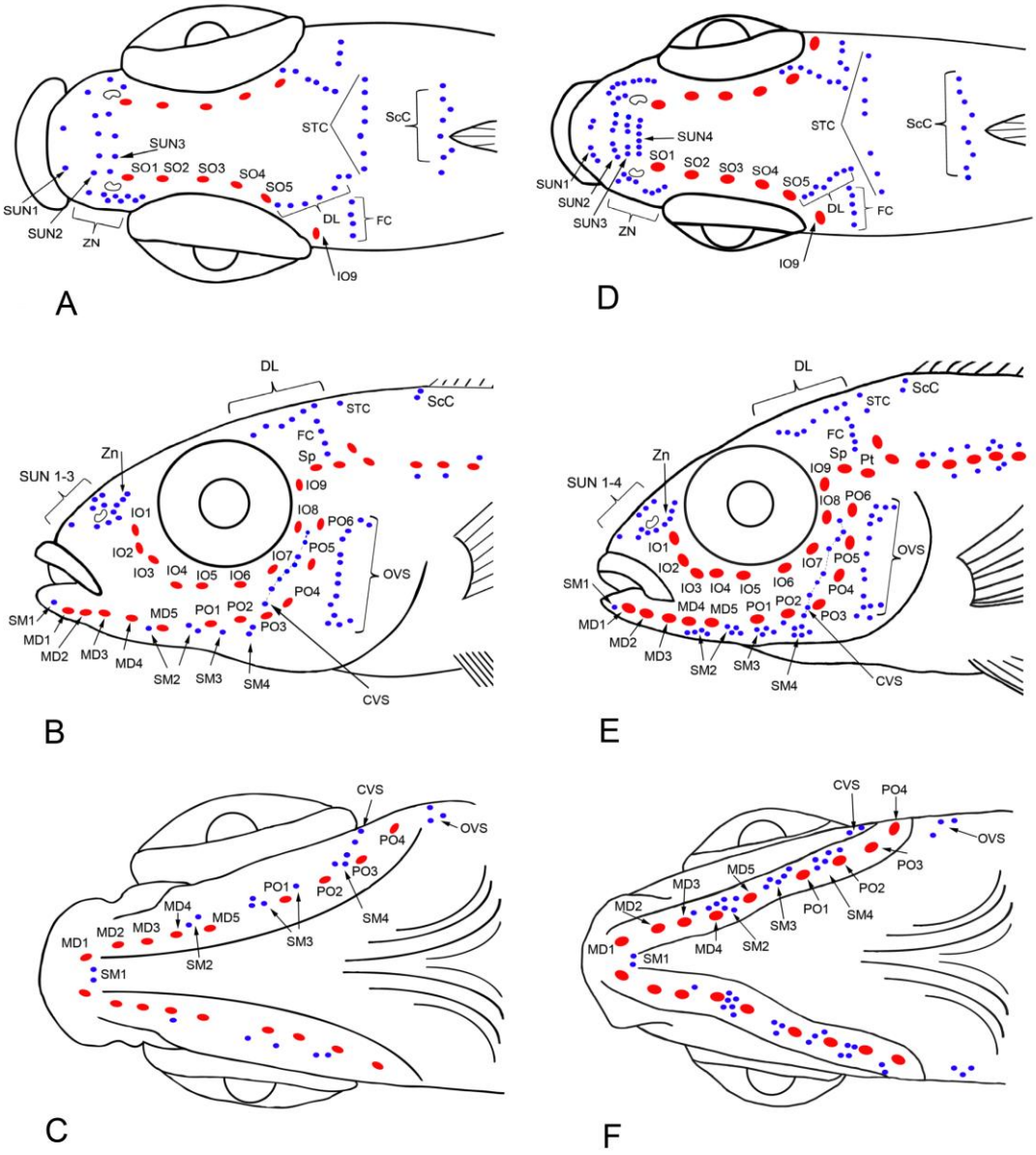

Fig. 1.4 . Canal (CN) and superficial (SN) neuromast names and distributions in dorsal, lateral and ventral views in *Tramitichromis* sp. (A-C) and *A. stuartgranti* (D-F). NM distributions are based on fluorescent images of 5 adult individuals of *Tramitichromis sp*.(narrow canals; 87-158 dpf;15-20 mm SL) and 6 adult individuals of *A. stuartgranti stuartgranti* (widened canals; 99-154 dpf; 16-26 mm SL). CNs are represented by large red dots/ovals (CNs in *A. stuartgranti* are larger than those in *Tramitichromis*) and SNs are represented by the smaller blue dots (drawn larger than actual size). Neuromast naming is based on Peters (1973, *Zeit. Morph Tiere*). See Table 1 for naming abbreviations.

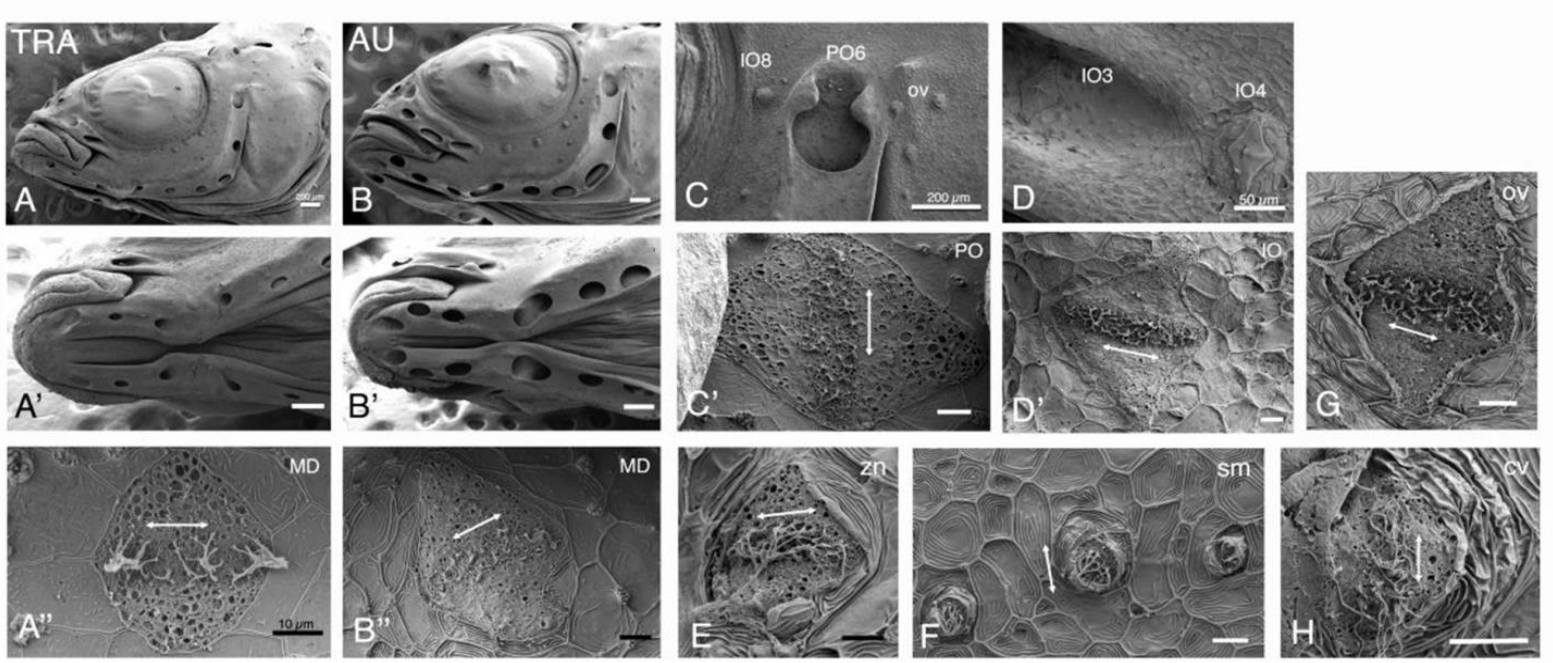

Fig.1.5 . SEM of canals, pores, and neuromasts in juvenile *Tramitichromis* sp. (A) and *A. stuartgranti* (B-H). A) *TRA* (10 mm SL, lateral and ventral views) and MD neuromast. B) *AU* (~11.5 mm SL, lateral and ventral views) and MD neuromast. C) AU PO neuromast in canal groove (11 mm SL). D) AU IO3 and IO4 CNs, and enlargement of representative IO4 CN. E-H) Superficial neuromasts in AU (see table 1 for abbreviations). Double headed arrows = hair cell orientation. Rostral is to left in all images. Scale bars: A, A', B, B', C = 200µm, D = 50 µm, all individual neuromast images = 10µm.

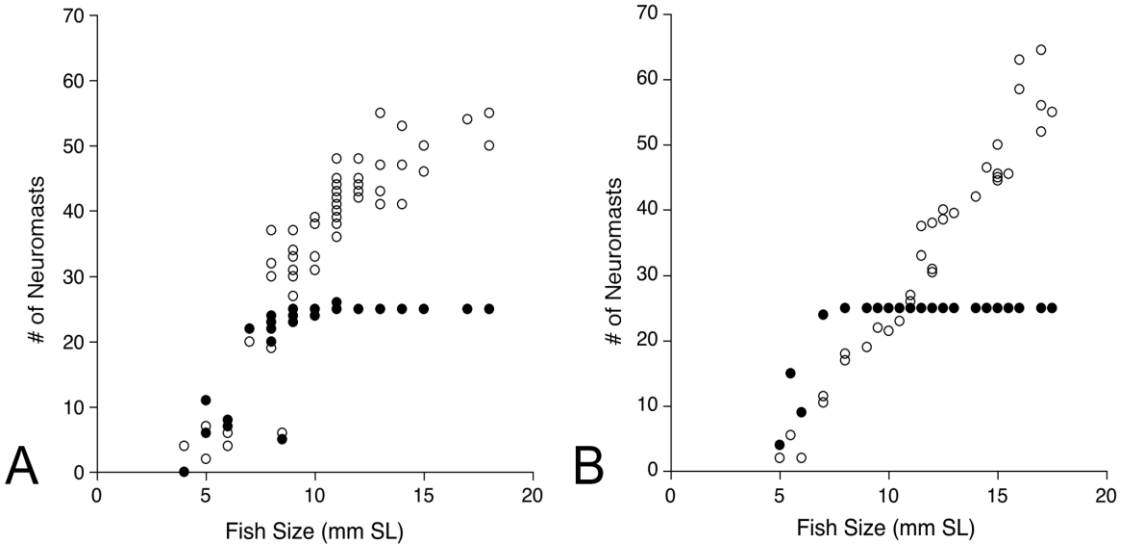

Fig. 1.6 . Ontogenetic increases in total canal neuromasts (CN, black circles) and superficial neuromasts(SN, white circles) in larvae and juveniles of *Tramitichromis* sp. (A, n=61) and *A. stuartgranti* (B, n=33). Note the stabilization of CN number and the continual increase of SN in both species.

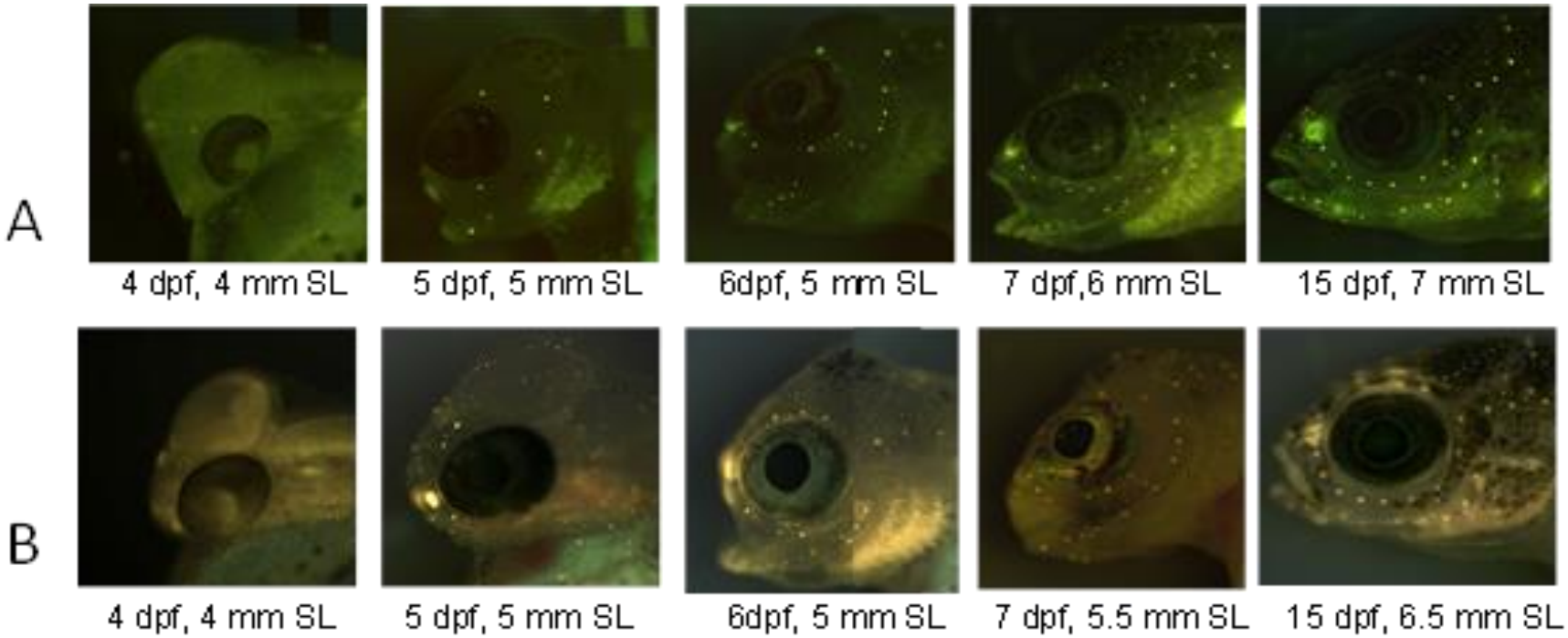

Fig.1.7 . Distribution of increasing number of superficial and large presumptive canal neuromasts in larvae and early juveniles (4-15 dpf, 4-7 mm SL) of A) *Tramitichromis* sp. (narrow canals) and B) *A. stuartgranti* (widened).

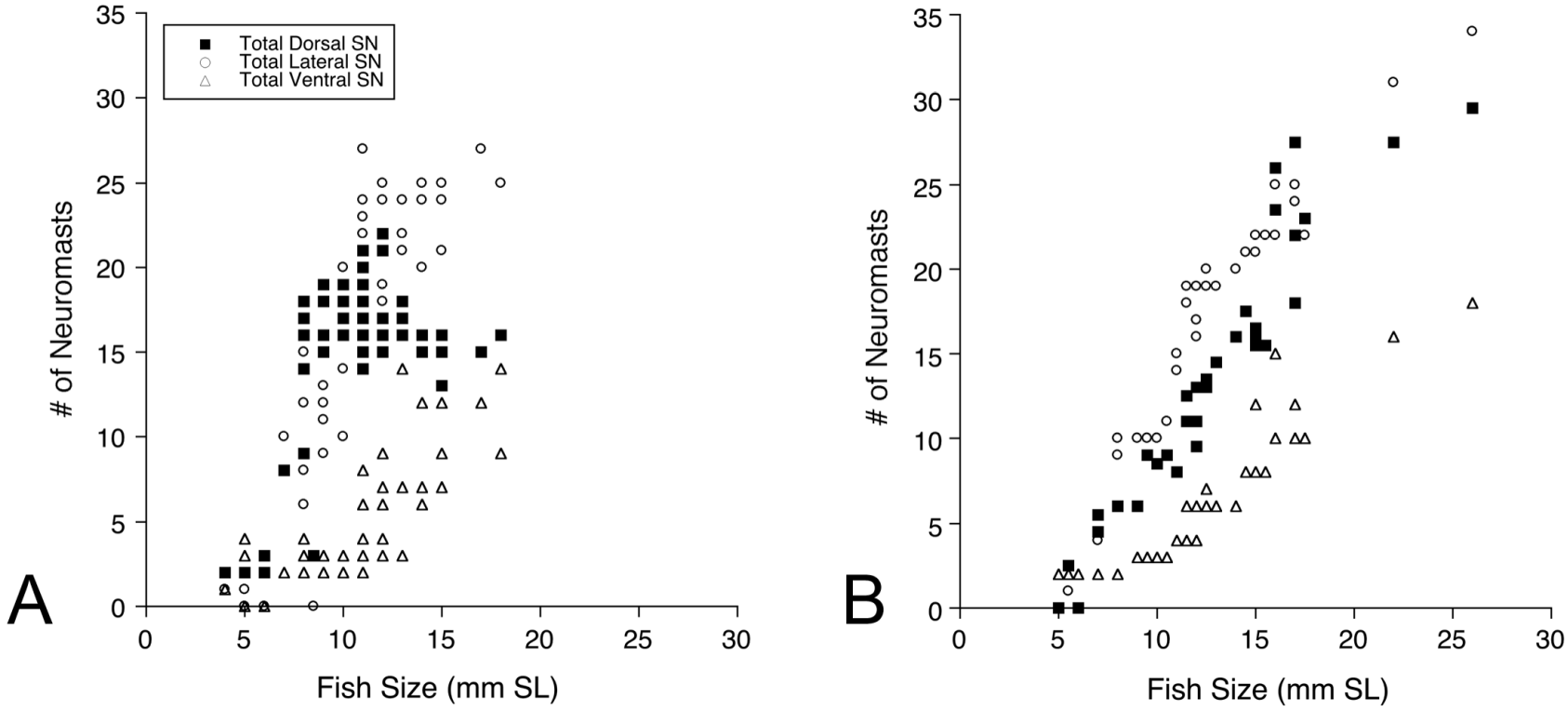

Fig. 1.8 . Ontogenetic increases in dorsal, ventral, and lateral superficial groups of NM (See Table 1 for identity of SN groups) for A)*Tramitichromis* sp. and B) *A. stuartgranti.*

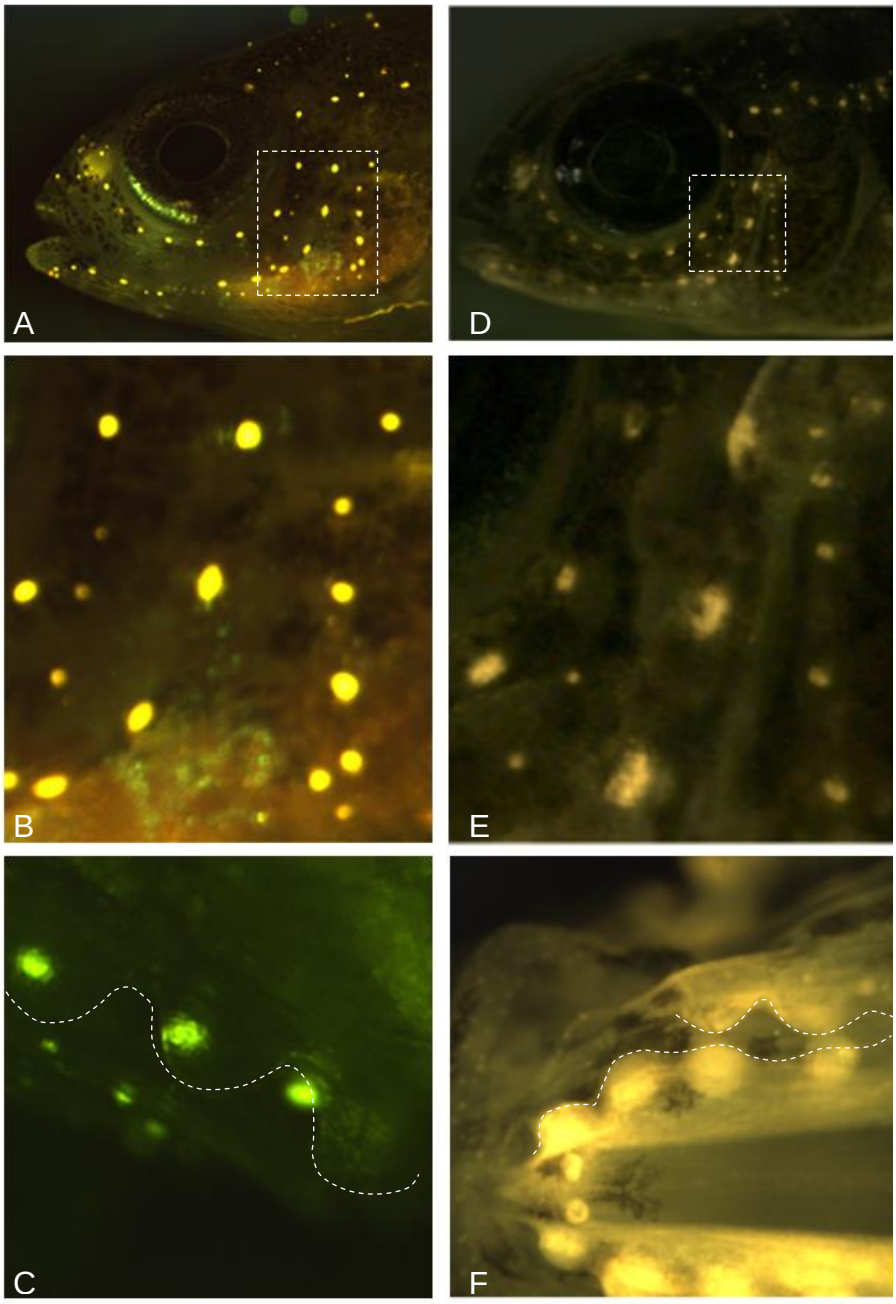

Fig.1.9 . Canal and superficial neuromasts in A-C) *Tramitichromis* sp. (10 mm SL, 12 dpf) and D-F)*A. stuartgranti* (15 mmSL, 40 dpf). Dashed boxes in A and D represent the magnified B and E images. Note the size differences between superficial (smaller yellow dots) and canal (larger yellow dots) neuromasts(B and E). Images C and F represent "scalloping" of the canals walls above and around the canal neuromasts (large yellow dots) before they meet and fuse together to form canal segments. The dashed white line indicates the outline of the "scallops".

Manuscript-2

Prepared for Submission to Hearing Research

Susceptibility and Recovery of Canal and Superficial Neuromasts to Cobalt Chloride and Gentamicin Ablation in Two Lake Malawi Cichlid Fishes: A Comparative Study

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ABSTRACT

Chemical and pharmacological ablation of neuromasts are methods frequently used to assess the impact of an inactive lateral line system on behavior. Fluorescent staining of neuromasts is also a common technique used to visually assess the effects of ablation on neuormasts. However, the two techniques have only been used in the same study once before to verify that lateral line ablation occurred and that behavior was a reflection of this. The effectiveness of these methods among treatments and how they affect superficial and canal neuromasts is uncertain. The current study provides the first detailed description of the effects of Cobalt (II) chloride heptahydrate as well as Gentamicin on superficial and canal neuromasts in two closely related cichlid species, *Tramitichromis* sp. and *A. stuartgranti*, using fluorescence staining (4-Di-2-ASP), as a method that can be used to verify the results of lateral line behavioral studies. Following treatment, it was determined that: 1) $CoCl_2$ in Ca^{++}-free water and Gentamicin had comparable effects on SNs and CNs in both species, 2) Treatment with $CoCl_2$ in Ca^{++}-free water and Gentamicin resulted in full recovery of both superficial and canal neuromasts by Day 4 or 7, 3) Treatment with $CoCl_2$ in tank water with Ca^{++} had no effect on SNs and CNs fluorescence on Day 0, when compared to $CoCl_2$ in Ca^{++}-free water and Gentamicin, and 4) Gentamicin does, in fact, ablate SNs, which refutes published reports.

INTRODUCTION

The lateral line system of fishes is composed of a series of mechanoreceptors called neuromasts, which are found on the trunk and head in specific patterns (reviewed by Coombs et al. 1988). The two types of neuromasts are superficial and canal neuromasts. Superficial neuromasts are located on the surface of the skin and are sensitive to water velocity, while canal neuromasts are in fluid-filled canals and detect water flow accelerations (Coombs et al. 2001). Neuromasts are made up of hair cells, each of which has one long kinocilium and many shorter stereocilia on its apical surface. Within a neuromast the hair cells are located above and in between non-sensory support cells, which are then surrounded by mantle cells. The ciliary bundles of the hair cells are physiologically polarized based on the position of the stereocilium relative to the kinocilia (Kasumyan, 2003). Each bundle projects into a single gelatinous cupula. The neuromasts of the lateral line system detect hydrodynamic flows arising from biotic and abiotic sources, and mediate several behaviors such as rheotaxis (Dijkgraaf, 1963; Kanter and Coombs, 2002) and prey detection (Hoekstra and Janssen, 1985; Montgomery and Coombs, 1998; Blaxter and Fuiman, 1989; Coombs et al., 2001).

Canal neuromasts are contained within cranial lateral line canals located in a conserved subset of dermal bones in the head. Among bony fishes, these canals may be narrow, widened, branched, or reduced (Webb, 1989b). Narrow canals, which are most common among fishes, are well-ossified and are perforated by pores in precise locations. In contrast, widened canals, which have evolved convergently in a small number of fish families (Webb, 1989a) have bony canal walls, but largely unossified canal roofs and the canals tend to be wider in diameter. The canal neuromasts are located beneath thin bony

bridges and large bony pores are in the canal roof, which is covered by an epithelium that is pierced by very small pores that connect the fluid inside the canal with the external environment.

Chemical and pharmacological ablation of neuromasts are methods frequently used to assess the impact of an inactive lateral line system on behavior. These techniques are commonly used on model species such as zebrafish and Mexican blind cave fish (Van Trump et al., 2010; Buck et al., 2012). Fluorescent staining of neuromasts is also a common technique used to visually assess the effects of ablation on neuormast. The two techniques, however have only been used sequentially once before to verify that lateral line ablation occurred and that behavior was a reflection of this (Schwalbe et al., 2012).

Cobalt (II) chloride heptahydrate (Co^{2+}) has been shown to block the mechanoreceptor channels of hair cells in neuromasts of fish (Baumann & Roth, 1986; Karlsen and Sand, 1987) rendering the fish unresponsive to water flows. Using the roach, Karlsen and Sand (1987) found the effects of cobalt in low Ca^{++} were reversed (evaluated using a behavioral reaction to a vibrating sphere) after 2-3 weeks, by placing them in normal tank water with an increased concentration of Ca^{++} .

Aminoglycoside antibiotics selectively block hair cell transduction channels including Ca^{++} channels (Hudspeth and Kroese, 1983; Kroese et al., 1989; Pichler et al. 1996). As in the inner ear, neuromast hair cells are mechanoreceptors for whose function the opening of Ca^{++} channels is essential. The effects of aminoglycosides on lateral line function have been assessed visually and behaviorally (Montgomery et al., 1997; Coombs et al., 2001). The effects of gentamicin on superficial and canal neuromasts in the Mexican blind cavefish (*Astyanax mexicanus*) and zebrafish (*Danio rerio*) has been

investigated, finding that both types of neuromasts are inactivated (Van Trump et. al. 2010).

Hair cells can be visualized *in vivo* using fluorescent styryl dyes, like DASPEI (2-(4-(dimethylamino)styryl)-N-ethylpyridinium iodide) , FM1-43 ((n-(3triethylammoniumpropyl)-4-(4-(dibutylamino)styryl) pyridinium dibromide), and 4-Di-2-ASP. The stain FM1-43 labels hair cells (but not support cells) by entering the mechanotransduction channels of stereocilia. When the mechanotransduction channels are unable to open or are blocked, this dye fails to travel into the hair cells and they are not stained (Meyers et al., 2003). The stain DASPEI labels mitochondria in live cells (Rafael, 1980) and thus preferentially labels the mitochondria-rich hair cells of neuromasts and the nasal sensory epithelium. DASPEI is thought to be taken up through the hair cell mechanotransduction channels, but if this channel is blocked, the mitochondria cannot be stained. DASPEI has been reported to label not only hair cells in superficial and canal neuromasts, but also support cells in zebrafish larvae (Harris et al., 2003). The stain 4-Di-2-ASP works similarly to DASPEI by entering the hair cell mechanotransduction channels and staining mitochondria (Magrassi et al., 1987) in hair cells and support cells of superficial and canal neuromasts.

This study is the first to provide a side- by- side comparison of lateral line ablation methods using cobalt (II) chloride heptahydrate and gentamicin in order to directly compare their immediate effects as well as the timing of recovery. There is ambiguity among ablation treatments and how they differentially affect superficial and canal neuromasts. Most ablation studies have been conducted on fish with narrow lateral line canals (zebrafish, Chiu et al., 2008; Coffin et al., 2009; Harris et al., 2003; Ou et al.,

2007; Owens et al., 2007; Van Trump et al., 2010; Buck et al., 2012; Mexican blind cave fish, Van Trump et al., 2010; Buck et al., 2012). The two species used in this study represent narrow (*Tramitichromis*) and widened (*A. stuartgranti*) canal morphologies, two of the four canal morphologies among teleosts. Narrow canal neuromasts tend to be smaller in size (with fewer hair cells) compared to widened canal neuromasts, and there is a possibility that differences in neuromast size may result in different susceptibility to one or both ablation methods. This study aimed to provide visual verification of neuromast function that can be used to interpret feeding behavior studies in which the lateral line system is ablated (Schwalbe et. al, 2012).

MATERIALS AND METHODS

Tramitichromis sp. and *A. stuartgranti*, mouth-brooding cichlids endemic to Lake Malawi (Africa), are commercially available and easily reared in a laboratory setting. Breeding tanks in two flow-through systems were lined with a mixture of sand and gravel, provided with mechanical and biological filtration, and kept at 80 ±1°F and salinity of 1±0.5 ppt with a 12:12 hour light: dark cycle. A breeding group of one male and several females were provided with PVC pipe, and rocks to mimic their natural habitat in order to promote breeding. Animals were fed daily on a varied diet (protein pellets, live brine shrimp, a pea/shrimp mixture, or an algae/yolk/earthworm protein flake mixture). The date on which a brood was noticed (e.g., expanded buccal cavity observed in female) was recorded, an indication that fertilization had occurred within 24 hours. Broods of yolk sac larvae (between 4 and 8 dpf) were removed from the mouth of brooding females and raised in small, round-bottomed glass flasks with slow water

exchange within small tanks in an Aquatic Habitats recirculating rack system. After yolk-sac absorption, actively swimming larvae swam out of the flasks, or were removed from the flask, and were then fed a flake mixture of egg yolk, earthworm protein, and algae.

Comparison of Vital Fluorescent Stains

In order to determine the most effective vital fluorescent stain for this study, a total of nine *A. stuartgranti* (12-16 mm SL) were vitally stained using one of three dyes as follows: 1) live fish were immersed in a 0.01% DASPEI solution (Sigma Aldrich) in tank water (Rubel lab, http://depts.washington.edu/rubelab) for 30 minutes, or 2) live fish were immersed in a 63 µM 4-Di-2-ASP (Sigma Aldrich) solution in tank water (based on a dose response experiment using *A. stuartgranti* ; doses tested were:15.8µM, 31.5µM, 47.3µM, and 63 µM [Nakae et al., 2011]; Fig. 1) for 5 minutes, or 3) live fish were immersed in a 3µM FM1-43 (Sigma Aldrich) solution in tank water for 45 seconds and rinsed with DI water (Santos, 2006).

After treatment with one of the three stains, each fish was anesthetized with MS-222 and immobilized in a petri dish for viewing. In a darkened room, a Nikon microscope (Model SMZ1500) was used to capture both light and fluorescent images on the head using a Spot digital camera (Model 25.22 Mp Color Mosaic) and Spot software (v. 5.0; Diagnostic Instruments, Sterling Heights, MI). Dark pigmentation prevented visualization of the canals in dorsal and lateral views. However, pigmentation over the mandibular canal was minimal, so all images were captured in a ventral orientation, which allowed for a side-by-side comparison of fluorescent staining of canal neuromasts and superficial neuromasts (MD 1 -5; SM1-4) in all fish. All three stains produced comparable results.

After weighing cost and exposure time needed, it was decided that 4-Di-2-ASP would be used for all subsequent experiments (Fig. 2).

Experiment # 1: Cobalt Chloride Ablation

The effect of cobalt chloride on canal (CNs) and superficial (SNs) neuromasts was assessed by comparing fish treated with $CoCl_2$ and control fish of *Tramitichromis* sp. in normal tank water or in Ca^{++} - free water. The Ca^{++} content of the Ca^{++} - free water was $\leq$ 20 mg/L, the lowest recordable level measured with a Hach Test Kit (Hach, Model HA-4P, Loveland, CO). This water , made up of chemicals that do not contain Ca^{++} ($MgSO_4$, $NaHCO_3$, NaCl, KCl, and KI in DI water) is described as Ca^{++} - free water here, to distinguish it from "tank water with Ca^{++}", which had 30 times the Ca^{++} content (660 mg/L). During preliminary trials (Fall 2011), the effects of a 0.05 mM and a 0.1 mM cobalt chloride solution were tested on *A. stuartgranti* with and without calcium in the water. The results indicated that immersion in a 0.1 mM solution resulted in strong ablation (elimination of hair cell fluorescence).

In order to determine the immediate effects of treatment and the timing of recovery from treatment, groups of 12 *Tramitichromis* sp. (12-16 mm SL) were subjected to one of two treatments or their appropriate control for three hours: 1) 0.1 mM Cobalt (II) Chloride in low calcium water solution (Kocher Lab protocol: $MgSO_4$ (7.9 g), $NaHCO_3$(15.7 g), NaCl (78.0 g), KCl (17.7 g), and KI (0.57 g) in 100 liters of DI water; calcium hardness=20 mg/L) or low calcium water (control; Kocher Lab protocol: $MgSO_4$ (7.9 g), $NaHCO_3$(15.7 g), NaCl (78.0 g), KCl (17.7 g), and KI (0.57 g) in 100 liters of DI water; calcium hardness$\leq$ 20 mg/L), or 2) 0.1 mM Cobalt (II) Chloride solution in normal

tank water (with Ca^{2+}; calcium hardness=660 mg/L) or tank water control (with Ca^{2+}; calcium hardness=660 mg/L). After treatment, the four groups of fish were housed in separate recovery tanks with normal tank water similar to what they had been reared in (Calcium Hardness=660 mg/L). On each of four days (0, 2, 4, and 7 days post treatment), three fish were sequentially stained with 63 µM 4-Di-2-ASP in tank water for 5 minutes. Each fish was then transferred to a solution of 0.04% MS-222 for anesthetization and immobilization with pins in a Sylgard-lined Petri dish for imaging (see Fig. 3).

This experiment was then repeated with *A. stuartgranti* (12-16 mm SL). Due to small brood sizes, some of the control *A. stuartgranti* were from a preliminary trial of the experiment carried out in summer 2012 were used here, but all *A. stuartgranti* exposed to $CoCl_2$ were from the experiment described above.

Experiment #2 : Gentamicin Ablation

The effect of gentamicin on both canal and superficial neuromasts was assessed in the two species. The procedure from Van Trump et al. (2010) was used as a guide for gentamicin concentration and exposure time. The effects of 0.01% (as used by Van Trump et al., 2010), 0.02%, 0.03%, and 0.04% gentamicin in tank water were tested on *A. stuartgranti* (See Fig. 4). A 0.04% solution of gentamicin was determined to have the strongest ablation results. The effect of time of exposure to gentamicin was tested using a 0.04% gentamicin solution to confirm that 24-hour exposure (as used by Van Trump et al., 2010) was sufficient to achieve complete neuromast ablation, as defined here by reduction in neuromast fluorescence in *Tramitichromis* sp. and *A. stuartgranti* (see Fig. 5).

In order to determine the immediate effects of gentamicin treatment and the timing of recovery, as reflected by the return of fluorescence, groups of 12 *Tramitichromis* sp. (12-16 mm SL) were treated with 0.04% gentamicin sulfate solution in tank water for 24 hours, or placed in tank water (control) for 24 hours. The two groups of fish were housed in separate tanks with normal tank water similar to their rearing water. Then on each of four days (0, 2, 4, and 7 days post-treatment), three treatment and three control fish were stained with a 63 µM 4-Di-2-ASP in tank water solution for 5 minutes. Fish were then transferred to a solution of MS-222 for anesthetization and immobilized with pins in a Sylgard-lined Petri dish for imaging (see Fig. 6). This experiment was then repeated with *A. stuartgranti* (12-16 mm SL).

Fluorescent Imaging and Scoring

Both light and fluorescent images of the mandibular canal neuromasts (MD 1-5) and mandibular superficial neuromasts (SM series, see Chapter 1) were captured using a Nikon dissecting microscope (Model SMZ1500) equipped with a Spot digital camera (Model 25.22 Mp Color Mosaic) and Spot software (v. 5.0; Diagnostic Instruments, Sterling Heights, MI). Each fluorescent image was taken at the same magnification (2x) and an exposure of 2048 msec to facilitate comparisons among individuals, among treatments, and between species. Fluorescent images were then used to assess the fluorescence of superficial and canal neuromasts on the mandible in all fish using all three ablation methods on Day 0, 2, 4, and 7 days post-treatment. A scoring system similar to that used by Van Trump et al. (2010) in their analysis of blind cavefish and zebrafish was used, but different methods were necessary to analyze fluorescence in

canal and superficial neuromasts. The ten mandibular neuromasts (MD 1-5; left and right) were scored as 0, 1, or 2 (based on where each CN is located; see Chapter 1), where 0 = no hair cell fluorescence, 1 = partial hair cell fluorescence (< 80%, judged visually against the control in a dark room similar to the conditions under which the images were captured), and 2 = full (normal) hair cell fluorescence (Fig. 7). The scores of the ten neuromasts in each fish, with three fish per treatment or per control (for a total of 30 canal neuromasts) were found to be normally distributed using the Shapiro-Wilk test for normality. Using a one way-ANOVA, no significant variation was found among the 3 fish on each day in each treatment. Therefore, the mean fluorescence score was calculated for all three fish (30 canal neuromasts) on each day for each treatment (Prism v. 6.0 for Windows, GraphPad Software, La Jolla, CA USA, www.graphpad.com).

In contrast to CNs, the mandibular SNs occur in several lines or clusters (See Chapter 1), vary in number among individuals and with fish size, and are not always found in the exact same locations, so the absence of fluorescence could not be recorded. Thus, we scored SNs as either 1or 2 (as defined above), but could not score neuromasts as 0 (absence of fluorescence). The scores of all superficial neuromasts for each of the three fish in all three treatments and their controls were found to be normally distributed using the Shapiro-Wilk test for normality. Using a one way-ANOVA, no significant variation was found among the 3 fish on each day in each treatment. Therefore, the mean fluorescence score was calculated for all of the superficial neuromasts in all three fish on each day for each treatment (Prism v. 6.0 for Windows, GraphPad Software, La Jolla, CA USA, www.graphpad.com).

The experimental design permitted a test of the immediate effect of cobalt chloride and gentamicin on SNs and CNs at Day 0, and with reference to the course of recovery (Day 0, 2, 4, and 7). On Day 0 and over the 7 day recovery period, the mean fluorescence score between the three treatments and between the three treatments and their controls, were analyzed using a two-way ANOVA followed by Tukey's multiple comparisons test (two independent variables: Treatment and Day; one dependent variable: Fluorescence Score; Prism v. 6.0 for Windows, GraphPad Software, La Jolla, CA USA,www.graphpad.com).

Figures were prepared using GraphPad Prism 6 and Adobe Illustrator CS5. Photographic images were cropped and arranged into plates using Adobe Photoshop CS5.

RESULTS

Both mandibular canal neuromasts (CNs) and the much smaller mandibular superficial neuromasts (SNs) were strongly labeled with 4-Di-2-ASP and were visible in the canal series and on the epithelium overlying the canal in all control fish (Fig. 11, 12, 13, 14). All three treatments ($CoCl_2$ in Ca^{++} - free water, $CoCl_2$ in tank water with Ca^{++}, and gentamicin in tank water) resulted in reduced CN and SN fluorescence (weaker staining of hair cells and the absence of staining in a subset of hair cells within a neuromast) on one or more days (Fig. 8, 11, 12, 13, 14). Both CNs and SNs in fish treated with $CoCl_2$ in Ca^{++} - free water and with gentamicin demonstrated reduced fluorescence on Day 0 (Fig. 11, 12, 13, 14) with an increase in fluorescence over the 7 day recovery period (Fig. 9, 10; Tables 1-4). In contrast, fish treated with $CoCl_2$ in tank water with Ca^{++} had variable fluorescence scores on Day 0 that were not different from their controls

(Fig. 9; Fig.10), with the exception of the SNs in *A. stuartgranti* (Fig. 10E), which showed reduced scores on Day 0 and a clear increase in fluorescence score over 7 days.

Comparison of Treatments on Day 0

All three treatments produced reduced neuromast fluorescence scores on Day 0 when compared to their controls (Fig. 8; Tables 5-8). However, $CoCl_2$ in Ca^{++} - free water and Gentamicin were significantly more effective (i.e. with lower fluorescence scores) than $CoCl_2$ in tank water with Ca^{++} on Day 0 (Tables5-8).

In *Tramitichromis*, $CoCl_2$ in Ca^{++} - free water had a significantly greater effect, as indicated by lower fluorescence, on SNs and CNs than $CoCl_2$ in tank water with Ca^{++} on Day 0. In *Tramitichromis*, $CoCl_2$ in Ca^{++} - free water and gentamicin had similar effects on CN and SN fluorescence scores on Day 0, but CN and SN in gentamicin treated fish had significantly lower fluorescence scores on Day 0 than those treated with $CoCl_2$ in tank water with Ca^{++} (see Table 5 for SN statistics; see Table 6 for CN statistics).

In *A. stuartgranti* , fish treated with $CoCl_2$ in Ca^{++} - free water had significantly lower fluorescence scores for CNs than those treated with $CoCl_2$ in tank water with Ca^{++} on Day 0. However, there was no significant difference in fluorescence scores for SNs in *A. stuartgranti* . $CoCl_2$ in Ca^{++} - free water and gentamicin had similar effects on SN and CN fluorescence scores on Day 0. Gentamicin treated *A. stuartgranti* had significantly lower fluorescence scores for CNs than those treated with $CoCl_2$ in tank water with Ca^{++} on Day 0, but there was no reduction in fluorescence scores for SN on Day 0 (see Table 7 for SN statistics; see Table 8 for CN statistics).

High- magnification images of CN's in fish treated with cobalt chloride and gentamicin on Day 0 typically revealed four types of labeling: 1) hair cells labeled around the edge of the neuromast only, 2) hair cells labeled at the center of the neuromasts only, 3) hair cells labeled around the edge *and* at the center of the neuromast, or 4) no hair cells labeled (Fig. 15).

Recovery from Cobalt Chloride Ablation

Tramitichromis sp. treated with $CoCl_2$ in Ca^{++} - free water, CNs exhibited significantly lower fluorescence scores than did control fish on Days 0 and 2, but on Days 4 and 7, fluorescence had increased so that there was no significant difference in fluorescence score between CNs in treated and control fish, which indicated recovery (Fig. 9A; Fig. 11; see Table 5 for statistics). *Tramitichromis* SNs also exhibited significantly lower fluorescence scores than those in control fish on Days 0 and 2. As with the CNs, fluorescence returned on Day 4 so that there was no significant difference in fluorescence score between SNs in treated and control fish. However, in contrast to the CNs, the SNs in treatment and control fish demonstrated a difference in fluorescence score on Day 7 (Fig.10A; see Table 6 for statistics).

In *A. stuartgranti* treated with $CoCl_2$ in Ca^{++} - free water, the CNs exhibited significantly lower fluorescence scores than did control fish on Days 0, 2, and 4, but on Day 7 fluorescence increased so that there was no significant difference between treatment and control fish (Fig. 9 D; Fig. 12; see Table 7 for statistics). The SNs exhibited the same statistically significant results (Fig. 10 D; see Table 8 for statistics).

Tramitichromis sp. and *A. stuartgranti* treated with $CoCl_2$ in tank water with Ca^{++}did not show a decrease in neuromast fluorescence, and so these results were thus distinct from that obtained with fish treated with $CoCl_2$in Ca^{++} - free water.

In *Tramitichromis* sp., CN fluorescence scores were not significantly different in treated fish and control fish on Days 0, 2, and 4, but, on Day 7 scores were significantly lower in treatment fish when compared to control fish (Fig. 9 B; Fig.11; see Table 5 for statistics). The SNs did not exhibit significantly lower fluorescence scores than control fish on Days 0, 2, and 7. However, on Day 4 there was an unexplainable difference in fluorescence scores in treated and control fish (Fig. 10B; see Table 6 for statistics).

In *A. stuartgranti* treated with $CoCl_2$ in tank water with Ca^{++}, the CNs had the same fluorescence scores as those in control fish on Day 0, indicating ineffective ablation. However, there was a significant difference in fluorescence scores between treatment and control fish on Days 2 and 4 , which could not be explained (Fig. 9 E; Fig. 12; see Table 7 for statistics). There was no difference in fluorescence scores between CNs in treated and control fish on Day 7, which suggests recovery. The SNs exhibited significantly lower fluorescence scores than those in control fish on Day 0, 2, 4, and Day 7 (Fig. 10E; see Table 8 for statistics). Nevertheless, a trend of increasing fluorescence scores in treated fish was noted from Day 0 through Day 7 (Fig. 10E), suggesting that recovery might be occurring.

Recovery from Gentamicin Ablation

In both *Tramitichromis* sp. and *A. stuartgranti* , the CNs and SNs treated with gentamicin showed the same results. In *Tramitichromis* sp., the CNs exhibited

significantly lower fluorescence scores than did control fish on Days 0 and 2, but on Days 4 and 7 there was no significant difference in fluorescence scores between treated and control fish (Fig. 9C; Fig. 13; see Table 5 for statistics). The SNs in *Tramitichromis* sp. exhibited the same statistically significant results as CNs (Fig.10C; see Table 6 for statistics). Similarly, in *A. stuartgranti* , the CNs had significantly lower fluorescence scores than those in control fish on Days 0 and 2, but on Days 4 and 7 there was no significant difference between treated and control fish (Fig. 9F; Fig. 14; see Table 7 for statistics), indicating recovery. The SNs in *A. stuartgranti* exhibited the same statistically significant results as CNs (Fig. 10 F; see Table 8 for statistics).

DISCUSSION

Over the past few decades, there has been an ongoing debate over the use of chemical and pharmacological methods for lateral line ablation and their effects on lateral line-mediated behavior (Karlsen and Sand, 1987; Song et al., 1995; Baker and Montgomery, 1999; Janssen, 2000; Harris et al., 2003; Liao, 2006; Van Trump et al., 2010). Most investigators have used only a change in behavior as an indicator of successful neuromast inactivation after treatment with cobalt or aminoglycoside antibiotics (Karlsen and Sand, 1987; Baker and Montgomery, 1999; Janssen, 2000; Liao, 2006). Other investigators have used only visual verification of neuromast inactivation using fluorescent stains, but different methods have not been compared side-by-side (SEM, Song et al., 1995; DASPEI, Harris et al., 2003; DASPEI and FM1-43, Owens et al. ,2009; DASPEI and 4-Di-2-ASP, Van Trump et al., 2010; Aminoglycosides, Buck et

al. , 2012). Only one investigator has used both behavioral and visual verification of neuromast ablation ($CoCl_2$, Schwalbe et al., 2012).

The current study has clarified the effects of two ablation methods, one chemical ($CoCl_2$) and the other pharmacological gentamicin), using fluorescent staining as an indicator of neuromast (hair cell) survival post treatment (Harris et al., 2003). It is clear from the results presented here that it is possible to ablate the hair cells of both canal and superficial neuromasts with either cobalt chloride or gentamicin. This study is also the first to visually compare the effects of cobalt *and* gentamicin immediately after treatment and over a 7-day recovery period. More specifically, the current study has shown that: 1) $CoCl_2$ in Ca^{++} - free water and Gentamicin had comparable effects on SNs and CNs in both species, indicated by similarly low fluorescence scores on Day 0 (first visual comparison of cobalt and gentamicin side by side), 2) Treatment with $CoCl_2$ in Ca^{++} - free water and Gentamicin resulted in full recovery of both superficial and canal neuromasts by Day 4 or Day 7, as indicated by fluorescence scores that were similar to controls, 3) treatment with $CoCl_2$ in tank water with Ca^{++} did not result in effective ablation of SNs and CNs, as indicated by significantly higher fluorescence scores on Day 0, when compared to $CoCl_2$ in Ca^{++} - free water and Gentamicin, 4) SNs and CNs treated with $CoCl_2$ in tank water with Ca^{++} did not show a clear recovery over the 7 day recovery period (i.e. variation in fluorescence scores over the 7 days), and 5) Gentamicin does, in fact, ablate SNs, as revealed by low fluorescence scores after treatment on Day 0. These results help to clarify the conflicting results of previous studies.

Cobalt Chloride Ablation

Cobalt (II) chloride heptahydrate ablation has been used to ablate the lateral line system, although there have been reservations as to what degree neuromasts are inactivated, what concentration is appropriate for different species, and what influence Ca^{++} concentration in water has on the effectiveness of cobalt chloride (Baker and Montgomery, 1999; Janssen, 2000; Montgomery et. al, 1997; Karlsen and Sand, 1975). Janssen (2000) discussed the effects of $CoCl_2$ concentration on hair cell inactivation and that interspecies differences may occur. He also indicated that using $CoCl_2$ concentrations higher than those suggested by Karlsen and Sand (1987; i.e. 0.1 mM for 12-24 hours) may be toxic to fish and would affect their overall health and behavior. Montgomery (1997) and Baker and Montgomery (1999) used a 2 mM solution of $CoCl_2$ for 3 hours to inactivate the lateral line system in Mexican blind cave fish. Janssen (2000) suggested that their behavioral data might have been due to $CoCl_2$ toxicity instead of a true response to $CoCl_2$ neuromast ablation. For this reason, and as a result of preliminary experiments, a 0.1 mM $CoCl_2$ solution was used so that toxicity did not play a role in hair cell inactivation.

The results of the current study support the findings of Karlsen and Sand (1987) that a lower concentration of Ca^{++} in the water during treatment with $CoCl_2$ results in stronger reduction of fluorescence, and that lower concentrations of Ca^{++} lead to longer recovery time of hair cells after treatment. A stronger ablation effect was indicated by lower CN and SN fluorescence scores on Day 0 (day of treatment) for fish treated with $CoCl_2$ in Ca^{++} - free water when compared to those treated with $CoCl_2$ in tank water with Ca^{++}. The only exception to this was for SNs in *A. stuartgranti* treated in $CoCl_2$ in Ca^{++} - free water

and $CoCl_2$ in tank water with Ca^{++}, where statistically similar fluorescence scores were obtained on Day 0. An explanation for this is not obvious, but it is possible that high levels of Ca^{++} in treatments with $CoCl_2$ in tank water with Ca^{++} (>600 mg/L), in the current study, would ensure competition of Ca^{++} with $CoCl_2$ for access to hair cell mechanosensory channels. This idea was offered by Karlsen and Sand (1975) for whom a 0.1 mM $CoCl_2$ concentration combined with higher levels of calcium in the water (1 mM) resulted in variable reactions by fish to a mechanosensory stimulus, indicating that ablation had occurred in some of the fish, but not in others. This variation in behavior may explain why SNs in *A. stuartgranti* on Day 0 were affected by $CoCl_2$ in tank water with Ca^{++}, but SNs in *Tramitichromis* sp. were not (i.e. ablation occurred in some of the fish [*A. stuartgranti*], but not in others [*Tramitichromis* sp.]). In addition, Schwalbe et al. (2012) showed that a Ca^{++} concentration of 60 mg/L (which is 3 times the concentration used in the Ca^{++} free treatments in the current study) with 0.1 mM $CoCl_2$ was sufficient to reduce or eliminate feeding behavior in *A. stuartgranti* .

Recovery after treatment with $CoCl_2$ in Ca^{++} - free water is revealed by an increase in SN and CN fluorescence scores over the course of the 7-day recovery period. In *Tramitichromis* sp., both CNs and SNs were recovered by Day 4, as indicated by the return of fluorescence. In *A. stuartgranti* both SNs and CNs recovered from treatment with $CoCl_2$ in Ca^{++} - free water by Day 7, three days later than in *Tramitichromis*.

The difference in recovery time (4 days vs. 7 days) may be due to the fact that *A. stuartgranti* has much larger canal neuromasts than does *Tramitichromis* (see Chapter 1). The smaller canal neuromasts in *Tramitichromis* likely have fewer hair cells, which in turn may initially be affected by $CoCl_2$ in Ca^{++}free water over the three hour treatment

period, but they have fewer hair cells to regenerate or recover from $CoCl_2$ (i.e. Ca^{++} replaces $CoCl_2$ in hair cells), which would take less time. Since CNs in *A. stuartgranti* likely have more hair cells based on their larger size, they may require a longer time to replace $CoCl_2$ with Ca^{++} in mechanosensory channels in the numerous hair cells (recovery), or a longer time to regenerate new hair cells (Schwalbe et al., 2012).

In *Tramitichromis*, a clear increase in fluorescence scores after ablation with $CoCl_2$ in tank water with Ca^{++} was not observed, indicating that hair cells in CNs and SNs were not recovering from ablation. In *A. stuartgranti* this pattern was anomalous such that SNs in fish treated with $CoCl_2$ in tank water with Ca^{++} increased in fluorescence score over the 7-day recovery period. Since SNs were affected on Day 0, then it makes sense that regeneration and/or recovery of hair cells would occur over the 7 days, as with the hair cells in $CoCl_2$ in Ca^{++} - free water treatments. CNs in *A. stuartgranti* treated with $CoCl_2$ in tank water with Ca^{++} did not show this clear pattern of recovery like their SNs, and CNs had fluorescence scores similar to *Tramitichromis* sp., showing no recovery.

Gentamicin Ablation

Previous studies have used Scanning Electron Microscopy (SEM) to visualize neuromasts after treatment with aminoglycoside antibiotics (i.e. neomycin and gentamicin). Some of these studies illustrated the disruption of hair cells in CNs, but not SNs (Song et al., 1995). However, new evidence presented by Van Trump et al. (2010) and the results obtained in the current study, which used fluorescent staining (DASPEI and 4-Di-2-ASP, respectively), make it clear that gentamicin does have an effect on *both* CNs and SNs.

Gentamicin can inactivate hair cells in two ways: 1) by blocking the mechanotransduction channels (Hudspeth and Kroese, 1983; Kroese et al., 1989; Pichler et al. 1996), or 2) by causing the degeneration of cilia and subsequent hair cell death (Williams et al., 1987; Forge and Schacht, 2000). Both of these mechanisms disrupt hair cell function and prevent the uptake of 4-Di-2-ASP. However, SEMs would show only hair cell death, and would not reveal the blocking of mechanotransduction channels. Song et al. (1995) used SEMs to view CNs and SNs after treatment with gentamicin, but reported hair cell death in canal neuromasts only. In contrast, Van Trump et al. (2010) and this study show reduction or elimination of fluorescence staining in both canal and superficial neuromasts in Mexican blind cave fish, *A. stuartgranti* , and *Tramitichromis* sp., respectively, indicating that gentamicin may block the mechanotransduction channels and cause hair cell death in both CNs and SNs.

An important contribution of the current study is the analysis of the time course of recovery after treatment with $CoCl_2$ or gentamicin. Song et al. (1995) only looked at neuromast recovery using SEMs from gentamicin in fish 4 days post-treatment, which is the time at which our results indicate full recovery from gentamicin. Hair cells in CNs and SNs are constantly being added and regenerated, and newly formed SNs contain many more immature hair cells that are resistant to gentamicin (Rubel, 1978). So when Song et al. (1995) looked at SEMs at 4 days post treatment, the hair cells in the SNs may have already regenerated/recovered, or may have been unaffected due to immaturity (Murakami et al., 2003).

The results obtained in this study indicate that gentamicin has the same effect on both SNs and CNs. The recovery time of SNs and CNs is also comparable, suggesting

that by 4 days post treatment, neuromast hair cells have either regenerated or recovered from gentamicin exposure.

Comparison of Cobalt and Gentamicin Ablation Methods

$CoCl_2$ and gentamicin have been used in a number of lateral line ablation experiments, at a number of different concentrations, and on a number of different species (roach, Karlsen and Sand, 1987; oscar, Song et al., 1995; Mexican blind cave fish, Montgomery et al., 1997; Mexican blind cave fish, Baker and Montgomery, 1999; Mexican blind cave fish, Janssen, 2000; Coombs et al., 2001; zebrafish, Harris et al., 2003; rainbow trout, Liao, 2006; zebrafish, Owens et al., 2009; Mexican blind cave fish and zebrafish, Van Trump et al., 2010; zebrafish, Buck et al. , 2012; *A. stuartgranti stuartgranti*, Schwalbe et al., 2012). It is clear from the current study that a 0.1 mM $CoCl_2$ in Ca++ - free water solution (as suggested in Karlsen and Sand, 1987) and a 0.04% gentamicin solution (based on Van Trump et al., 2010) are the most effective ablation techniques to use on cichlids in the genera *Tramitichromis* sp. and *A. stuartgranti* .

It is important to note that in fish treated with $CoCl_2$ in Ca^{++} - free water, fluorescence scores for CNs on Day 0 were lower in *Tramitichromis* sp. than in *A. stuartgranti* , indicating that a larger proportion of hair cells had been ablated in *Tramitichromis* sp. In addition, neuromast recovery time was about 3 days longer in *A. stuartgranti* than *Tramitichromis* sp. It is possible that on Day 0, *A. stuartgranti* CNs, which are larger and presumably contain more hair cells than those in *Tramitichromis* sp., had fewer hair cells that were affected because there was not a long enough exposure

time to ablate all of them. It follows that *Tramitichromis* sp. has smaller neuromasts and presumably fewer hair cells, all or most of which were ablated, as indicated by a much reduced fluorescence score. The fact that *A. stuartgranti* fluorescence scores took 3 days longer to increase to the same fluorescence score as control fish, thus indicating recovery, when compared to *Tramitichromis* sp., may be due to the regeneration or repair of a larger proportion of hair cells in canal neuromasts.

On the other hand, gentamicin treatment had the same effects on fluorescence scores for SNs and CNs in both species on Day 0 and on subsequent recovery days. This might indicate that treatment with 0.04% gentamicin for 24 hours is a more appropriate ablation method to use on fish with widened canals to ensure complete ablation of their larger canal neuromasts in addition to their SNs. Gentamicin-treated fish did have CN and SN fluorescence scores that were similar to $CoCl_2$ in Ca^{++} - free water, so both methods likely have the same overall effect on behavior.

All previously published lateral line ablation experiments have been conducted on species with narrow canals (like *Tramitichromis*), which have relatively small canal neuromasts, or on species with a proliferation of superficial neuromasts (i.e. Mexican blind cave fish), which are also small in size and relatively low in hair cell number. An exception to this is the work of Schwalbe et al. (2012; *A. stuartgranti stuartgranti*).

Finally, the styryl dye 4-Di-2-ASP provided a rapid and largely reliable method for visually assessing the effects of both cobalt and gentamicin on neuromast hair cells in two species of cichlids with different lateral line canal morphologies. The results presented here show that verification of neuromast ablation using fluorescent staining is important for verifying behavioral experiments.

Table 2.1. The effect of cobalt chloride and gentamicin and on mandibular canal neuromasts in *Tramitichromis* sp. as revealed by 4-Di-2-ASP staining. Mean ±SE scores for staining of the 10 mandibular canal neuromasts (5 right, 5 left) in three treatments with controls on day of treatment (Day 0 and then Days 2, 4, 7). Score 0=no 4-Di-2-ASP staining, Score 1=partial staining, and Score 2=full normal staining. See Fig. 7 for examples of canal neuromasts scored as 0, 1, and 2.

Treatment	Day 0	Day 2	Day 4	Day 7
$CoCl_2$ 0.1mM in Ca^{++} -free tank water	0	1	2	1.5
	0.4	1	2	2
	0.4	0.5	2	2
	0.3 ±0.13	0.8 ±0.17	2.0 ±0.00	1.8 ±0.17
Ca^{++} -free tank water (Control)	2	2	2	1.9
	2	1.8	2	1.8
	2	1.7	2	2
	2.0 ±0.00	1.8 ±0.09	2.0 ± 0.00	1.9 ±0.06
$CoCl_2$ 0.1mM% in tank water with calcium	1.8	2	1.9	1.4
	1.7	1.4	1.7	1.8
	2	1	1.8	1.8
	1.8 ± 0.09	1.5 ±0.29	1.8 ±0.06	1.7 ±0.13
Tank water with calcium (Control)	1.9	1.9	2	2
	1.9	1.6	1.9	2
	2	1.5	2	2
	1.9 ±0.03	1.7 ± 0.12	2.0 ±0.00	2.0 ±0.00
Gentamicin 0.04% in tank water	0	1	2	2
	0.5	1	2	2
	0.3	1	2	2
	0.3 ± 0.15	1.0 ± 0.00	2.0 ±0.00	2.0 ± 0.00
Tank water (Control)	2	2	2	2
	2	2	2	2
	-	2	2	2
	2.0 ±0.00	2.0 ±0.00	2.0 ±0.00	2.0 ±0.00

Table 2.2. The effect of gentamicin and cobalt chloride on mandibular canal neuromasts in *A. stuartgranti.* Mean ± SE scores for staining of the 10 mandibular canal neuromasts (5 right, 5 left) in three treatments with controls on day of treatment (Day 0 and then Days 2, 4, 7). Score 0=no 4-Di-2-ASP staining, Score 1=partial staining, and Score 2=full normal staining. See Fig. 7 for examples of canal neuromasts scored as 0, 1, and 2.

Treatment	Day 0	Day 2	Day 4	Day 7
$CoCl_2$ 0.1mM in Ca^{++}-free tank water	0.7 0.4 0.9 0.7 ± 0.15	0.2 0.9 0.9 0.7 ± 0.23	1.1 1.5 1.4 1.3 ± 0.12	2 1.8 1.9 1.9 ± 0.06
Ca^{++}-free tank water (Control)	2 2 2 2.0 ±0.00	2 1.9 2 2.0 ± 0.03	2 2 2 2.0 ±0.00	2 2 1.7 1.9± 0.10
$CoCl_2$ 0.1mM% in tank water with calcium	1.8 1.8 1.4 1.7 ± 0.13	1 1.8 1.4 1.4 ±0.23	1.9 1 2 1.6 ± 0.32	2 2 2 2 ±0.00
Tank water with calcium (Control)	1.8 1.8 2 1.9 ± 0.07	2 2 - 2.0±0.00	2 2 - 2.0±0.00	2 2 - 2.0±0.00
Gentamicin 0.04% in tank water	0.2 0.2 0.2 0.2 ±0.00	1 1.8 1.3 1.4 ±0.23	2 2 2 2.0 ± 0.00	1.9 1.9 2 1.9 ± 0.03
Tank water (Control)	2 2 2 2.0 ± 0.00	1.9 1.9 2 1.9 ± 0.03	2 2 - 2.0 ±0.00	2 1.8 2 1.9 ± 0.07

Table 2.3. The effect of gentamicin and cobalt chloride on mandibular superficial neuromasts in *Tramitichromis* sp. Mean score of mandibular SNs scored as 1 or 2, three treatments with controls over 7 days. 1=partial staining and a score of 2=full normal staining. See Fig. 7 for examples of SNs scored as 1 and 2.

Treatment	Day 0	Day 2	Day 4	Day 7
$CoCl_2$ 0.1mM in Ca^{++}-free tank water	1.0 1.3 1.0 1.06 ±0.04	1.0 1.0 1.0 1.00 ±0.00	1.9 1.7 1.6 1.7 ±0.06	1.8 1.7 1.6 1.73 ±0.08
Ca^{++} -free tank water (Control)	1.8 1.7 2.0 1.76 ±0.08	1.8 1.7 1.5 1.73 ±0.08	1.8 1.9 1.9 1.83±0.069	2.0 1.9 2.0 1.97 ±0.03
$CoCl_2$ 0.1mM in tank water with Ca^{++}	2.0 1.8 1.8 1.9 ±0.06	2.0 1.8 1.9 1.9 ±0.07	1.2 1.0 1.3 1.07 ±0.05	1.9 2.0 1.5 1.93 ±0.05
Tank water with calcium(Control)	2.0 2.0 1.6 1.87 ± 0.06	1.9 2.0 1.9 1.93 ±0.05	1.6 1.8 1.5 1.67 ± 0.09	2.0 2.0 2.0 2.0 ± 0.00
Gentamicin 0.04% in tank water	1.0 1.0 1.0 1.00 ± 0.00	1.0 1.6 1.9 1.17 ±0.07	2.0 1.7 1.9 1.87 ±0.03	2.0 1.9 2.0 1.97 ±0.03
Tank water only (Control)	2.0 2.0 - 2.0 ± 0.00	1.9 2.0 2.0 1.97 ±0.06	1.9 1.6 1.9 1.8 ±0.07	1.8 1.9 2.0 1.87 ±0.07

Table 2.4. The effect of gentamicin and cobalt chloride on mandibular superficial neuromasts in *A. stuartgranti*. Mean score of mandibular SNs scored as 1 or 2, for three treatments with controls over 7 days. 1=partial staining and a score of 2=full normal staining. See Fig. 7 for examples of SNs scored as 1 and 2.

Treatment	Day 0	Day 2	Day 4	Day 7
$CoCl_2$ 0.1mM in Ca^{++}- free tank water	1.0 1.0 1.0 1.00 ±0.00	1.0 1.0 1.0 1.00 ±0.00	1.6 1.5 1.7 1.53 ±0.09	1.9 1.7 1.6 1.83 ±0.07
Ca^{++} -free tank water (Control)	2.0 2.0 2.0 2.00 ±0.00	2.0 1.9 2.0 1.96 ±0.033	2.0 2.0 2.0 2.00 ±0.00	2.0 1.9 2.0 1.96 ±0.033
$CoCl_2$ 0.1mM in tank water with Ca^{++}	1.0 1.0 1.1 1.03 ±0.03	1.3 1.0 1.0 1.20 ±0.07	1.7 1.4 1.6 1.46 ±0.09	2.0 2.0 1.5 1.76 ±0.08
Tank water with calcium(Control)	2.0 2.0 2.0 2.0 ± 0.00	2.0 2.0 - 2.0 ± 0.00	2.0 2.0 - 2.0 ± 0.00	2.0 2.0 - 2.0 ± 0.00
Gentamicin 0.04% in tank water	1.0 1.0 1.0 1.0 ± 0.00	1.5 1.1 1.5 1.40 ±0.09	1.9 1.9 1.7 1.86 ±0.06	1.8 1.8 2.0 1.73 ±0.08
Tank water only (Control)	1.3 1.6 1.8 1.46 ± 0.09	2.0 2.0 1.9 1.96 ±0.03	1.9 1.9 - 1.90 ±0.05	1.7 1.8 2.0 1.83 ±0.07

Table 2. 5. Summary statistics for $CoCl_2$ and gentamicin treated *Tramitichromis* sp. canal neuromast comparisons. * indicates a p-value <0.05.

	Significance	p- value
Day 0		
0.1mM $CoCl_2$in Ca^{++}free water vs. Ca^{++} - free water (Control)	*	< 0.0001
0.1mM $CoCl_2$in Ca^{++}-free water vs. 0.1 mM $CoCl_2$in tank water (w/ Ca^{++})	*	< 0.0001
0.1mM $CoCl_2$in Ca^{++}-free water vs. 63µM Gentamicin	ns	> 0.9999
Ca^{++} - free water (Control) vs. Tank water (w/ Ca^{++}, control)	ns	0.9633
Ca^{++} - free water (Control) vs. Tank water (Gentamicin control)	ns	> 0.9999
0.1 mM $CoCl_2$in tank water (w/ Ca^{++}) vs. Tank water (w/ Ca^{++}, control)	ns	0.8199
0.1 mM $CoCl_2$in tank water (w/ Ca^{++}) vs. 63µM Gentamicin	*	< 0.0001
Tank water (w/ Ca^{++}, control) vs. Tank water (Gentamicin control)	ns	0.9774
63µM Gentamicin vs. Tank water (Gentamicin control)	*	< 0.0001
Day 2		
0.1mM $CoCl_2$in Ca^{++}-free water vs. Ca^{++} - free water (Control)	*	< 0.0001
0.1mM $CoCl_2$in Ca^{++}-free water vs. 0.1 mM $CoCl_2$in tank water (w/ Ca^{++})	*	< 0.0001
0.1mM $CoCl_2$in Ca^{++}-free water vs. 63µM Gentamicin	ns	0.3112
Ca^{++} - free water (Control) vs. Tank water (w/ Ca^{++}, control)	ns	0.3112
Ca^{++} - free water (Control) vs. Tank water (Gentamicin control)	ns	0.3112
0.1 mM $CoCl_2$in tank water (w/ Ca^{++}) vs. Tank water (w/ Ca^{++}, control)	ns	0.1349
0.1 mM $CoCl_2$in tank water (w/ Ca^{++}) vs. 63µM Gentamicin	*	< 0.0001
Tank water (w/ Ca^{++}, control) vs. Tank water (Gentamicin control)	*	0.0006
63µM Gentamicin vs. Tank water (Gentamicin control)	*	< 0.0001
Day 4		
0.1mM $CoCl_2$in Ca^{++}-free water vs. Ca^{++} - free water (Control)	ns	> 0.9999
0.1mM $CoCl_2$in Ca^{++}-free water vs. 0.1 mM $CoCl_2$in tank water (w/ Ca^{++})	ns	0.1349
0.1mM $CoCl_2$in Ca^{++}-free water vs. 63µM Gentamicin	ns	> 0.9999
Ca^{++} - free water (Control) vs. Tank water (w/ Ca^{++}, control)	ns	0.9985
Ca^{++} - free water (Control) vs. Tank water (Gentamicin control)	ns	> 0.9999
0.1 mM $CoCl_2$in tank water (w/ Ca^{++}) vs. Tank water (w/ Ca^{++}, control)	ns	0.3112
0.1 mM $CoCl_2$in tank water (w/ Ca^{++}) vs. 63µM Gentamicin	ns	0.1349
Tank water (w/ Ca^{++}, control) vs. Tank water (Gentamicin control)	ns	0.9985
63µM Gentamicin vs. Tank water (Gentamicin control)	ns	> 0.9999
Day 7		
0.1mM $CoCl_2$in Ca^{++}-free water vs. Ca^{++} - free water (Control)	ns	0.9633
0.1mM $CoCl_2$in Ca^{++}-free water vs. 0.1 mM $CoCl_2$in tank water (w/ Ca^{++})	ns	0.3112
0.1mM $CoCl_2$in Ca^{++}-free water vs. 63µM Gentamicin	ns	0.3112
Ca^{++} - free water (Control) vs. Tank water (w/ Ca^{++}, control)	ns	0.8199
Ca^{++} - free water (Control) vs. Tank water (Gentamicin control)	ns	0.8199
0.1 mM $CoCl_2$in tank water (w/ Ca^{++}) vs. Tank water (w/ Ca^{++}, control)	*	0.0006
0.1 mM $CoCl_2$in tank water (w/ Ca^{++}) vs. 63µM Gentamicin	*	0.0006
Tank water (w/ Ca^{++}, control) vs. Tank water (Gentamicin control)	ns	> 0.9999
63µM Gentamicin vs. Tank water (Gentamicin control)	ns	> 0.9999

Table 2. 6. Summary statistics for $CoCl_2$ and gentamicin treated *Tramitichromis* sp. superficial neuromast comparisons. * indicates a p-value < 0.05.

	Significance	p- value
Day 0		
0.1mM $CoCl_2$in Ca^{++}free water vs. Ca^{++} - free water (Control)	*	< 0.0001
0.1mM $CoCl_2$in Ca^{++}-free water vs. 0.1 mM $CoCl_2$in tank water (w/ Ca^{++})	*	< 0.0001
0.1mM $CoCl_2$in Ca^{++}-free water vs. 63µM Gentamicin	ns	0.9624
Ca^{++} - free water (Control) vs. Tank water (w/ Ca^{++}, control)	ns	0.5620
Ca^{++} - free water (Control) vs. Tank water (Gentamicin control)	*	0.0125
0.1 mM $CoCl_2$in tank water (w/ Ca^{++}) vs. Tank water (w/ Ca^{++}, control)	ns	0.9984
0.1 mM $CoCl_2$in tank water (w/ Ca^{++}) vs. 63µM Gentamicin	*	< 0.0001
Tank water (w/ Ca^{++}, control) vs. Tank water (Gentamicin control)	ns	0.5620
63µM Gentamicin vs. Tank water (Gentamicin control)	*	< 0.0001
Day 2		
0.1mM $CoCl_2$in Ca^{++}-free water vs. Ca^{++} - free water (Control)	*	< 0.0001
0.1mM $CoCl_2$in Ca^{++}-free water vs. 0.1 mM $CoCl_2$in tank water (w/ Ca^{++})	*	< 0.0001
0.1mM $CoCl_2$in Ca^{++}-free water vs. 63µM Gentamicin	ns	0.3048
Ca^{++} - free water (Control) vs. Tank water (w/ Ca^{++}, control)	ns	0.1305
Ca^{++} - free water (Control) vs. Tank water (Gentamicin control)	ns	0.5620
0.1 mM $CoCl_2$in tank water (w/ Ca^{++}) vs. Tank water (w/ Ca^{++}, control)	ns	0.9984
0.1 mM $CoCl_2$in tank water (w/ Ca^{++}) vs. 63µM Gentamicin	*	< 0.0001
Tank water (w/ Ca^{++}, control) vs. Tank water (Gentamicin control)	ns	0.9624
63µM Gentamicin vs. Tank water (Gentamicin control)	*	< 0.0001
Day 4		
0.1mM $CoCl_2$in Ca^{++}-free water vs. Ca^{++} - free water (Control)	ns	0.9984
0.1mM $CoCl_2$in Ca^{++}-free water vs. 0.1 mM $CoCl_2$in tank water (w/ Ca^{++})	*	< 0.0001
0.1mM $CoCl_2$in Ca^{++}-free water vs. 63µM Gentamicin	ns	0.8162
Ca^{++} - free water (Control) vs. Tank water (w/ Ca^{++}, control)	ns	0.3048
Ca^{++} - free water (Control) vs. Tank water (Gentamicin control)	ns	0.9984
0.1 mM $CoCl_2$in tank water (w/ Ca^{++}) vs. Tank water (w/ Ca^{++}, control)	*	< 0.0001
0.1 mM $CoCl_2$in tank water (w/ Ca^{++}) vs. 63µM Gentamicin	*	< 0.0001
Tank water (w/ Ca^{++}, control) vs. Tank water (Gentamicin control)	ns	0.5620
63µM Gentamicin vs. Tank water (Gentamicin control)	ns	0.3048
Day 7		
0.1mM $CoCl_2$in Ca^{++}-free water vs. Ca^{++} - free water (Control)	*	0.0448
0.1mM $CoCl_2$in Ca^{++}-free water vs. 0.1 mM $CoCl_2$in tank water (w/ Ca^{++})	ns	0.1305
0.1mM $CoCl_2$in Ca^{++}-free water vs. 63µM Gentamicin	*	0.0448
Ca^{++} - free water (Control) vs. Tank water (w/ Ca^{++}, control)	ns	0.9984
Ca^{++} - free water (Control) vs. Tank water (Gentamicin control)	ns	0.5620
0.1 mM $CoCl_2$in tank water (w/ Ca^{++}) vs. Tank water (w/ Ca^{++}, control)	ns	0.9624
0.1 mM $CoCl_2$in tank water (w/ Ca^{++}) vs. 63µM Gentamicin	ns	0.9984
Tank water (w/ Ca^{++}, control) vs. Tank water (Gentamicin control)	ns	0.3048
63µM Gentamicin vs. Tank water (Gentamicin control)	ns	0.5620

Table 2. 7. Summary statistics for $CoCl_2$ and gentamicin treated *A. stuartgranti* canal neuromast comparisons. * indicates a p-value < 0.05.

	Significance	p-value
Day 0		
0.1mM $CoCl_2$in Ca^{++}-free water vs. Ca^{++} - free water (Control)	*	< 0.0001
0.1mM $CoCl_2$in Ca^{++}-free water vs. 0.1 mM $CoCl_2$in tank water (w/ Ca^{++})	*	< 0.0001
0.1mM $CoCl_2$in Ca^{++}-free water vs. 63µM Gentamicin	*	< 0.0001
Ca^{++} - free water (Control) vs. Tank water (w/ Ca^{++}, control)	ns	0.6601
Ca^{++} - free water (Control) vs. Tank water (Gentamicin control)	ns	> 0.9999
0.1 mM $CoCl_2$in tank water (w/ Ca^{++}) vs. Tank water (w/ Ca^{++}, control)	ns	0.2122
0.1 mM $CoCl_2$in tank water (w/ Ca^{++}) vs. 63µM Gentamicin	*	< 0.0001
Tank water (w/ Ca^{++}, control) vs. Tank water (Gentamicin control)	ns	0.6601
63µM Gentamicin vs. Tank water (Gentamicin control)	*	< 0.0001
Day 2		
0.1mM $CoCl_2$in Ca^{++}-free water vs. Ca^{++} - free water (Control)	*	< 0.0001
0.1mM $CoCl_2$in Ca^{++}-free water vs. 0.1 mM $CoCl_2$in tank water (w/ Ca^{++})	*	< 0.0001
0.1mM $CoCl_2$in Ca^{++}-free water vs. 63µM Gentamicin	*	< 0.0001
Ca^{++} - free water (Control) vs. Tank water (w/ Ca^{++}, control)	ns	> 0.9999
Ca^{++} - free water (Control) vs. Tank water (Gentamicin control)	ns	0.9750
0.1 mM $CoCl_2$in tank water (w/ Ca^{++}) vs. Tank water (w/ Ca^{++}, control)	*	< 0.0001
0.1 mM $CoCl_2$in tank water (w/ Ca^{++}) vs. 63µM Gentamicin	ns	0.9990
Tank water (w/ Ca^{++}, control) vs. Tank water (Gentamicin control)	ns	0.9848
63µM Gentamicin vs. Tank water (Gentamicin control)	*	< 0.0001
Day 4		
0.1mM $CoCl_2$in Ca^{++}-free water vs. Ca^{++} - free water (Control)	*	< 0.0001
0.1mM $CoCl_2$in Ca^{++}-free water vs. 0.1 mM $CoCl_2$in tank water (w/ Ca^{++})	*	0.0096
0.1mM $CoCl_2$in Ca^{++}-free water vs. 63µM Gentamicin	*	< 0.0001
Ca^{++} - free water (Control) vs. Tank water (w/ Ca^{++}, control)	ns	> 0.9999
Ca^{++} - free water (Control) vs. Tank water (Gentamicin control)	ns	> 0.9999
0.1 mM $CoCl_2$in tank water (w/ Ca^{++}) vs. Tank water (w/ Ca^{++}, control)	*	0.0031
0.1 mM $CoCl_2$in tank water (w/ Ca^{++}) vs. 63µM Gentamicin	*	0.0006
Tank water (w/ Ca^{++}, control) vs. Tank water (Gentamicin control)	ns	> 0.9999
63µM Gentamicin vs. Tank water (Gentamicin control)	ns	> 0.9999
Day 7		
0.1mM $CoCl_2$in Ca^{++}-free water vs. Ca^{++} - free water (Control)	ns	> 0.9999
0.1mM $CoCl_2$in Ca^{++}-free water vs. 0.1 mM $CoCl_2$in tank water (w/ Ca^{++})	ns	0.8689
0.1mM $CoCl_2$in Ca^{++}-free water vs. 63µM Gentamicin	ns	0.9990
Ca^{++} - free water (Control) vs. Tank water (w/ Ca^{++}, control)	ns	0.9144
Ca^{++} - free water (Control) vs. Tank water (Gentamicin control)	ns	0.9990
0.1 mM $CoCl_2$in tank water (w/ Ca^{++}) vs. Tank water (w/ Ca^{++}, control)	ns	> 0.9999
0.1 mM $CoCl_2$in tank water (w/ Ca^{++}) vs. 63µM Gentamicin	ns	0.9750
Tank water (w/ Ca^{++}, control) vs. Tank water (Gentamicin control)	ns	0.9848
63µM Gentamicin vs. Tank water (Gentamicin control)	ns	> 0.9999

Table 2. 8. Summary statistics for $CoCl_2$ and gentamicin treated *A. stuartgranti* superficial neuromast comparisons. * indicates a p-value < 0.05.

	Significance	p- value
Day 0		
0.1mM $CoCl_2$in Ca^{++}-free water vs. Ca^{++} - free water (Control)	*	< 0.0001
0.1mM $CoCl_2$in Ca^{++}-free water vs. 0.1 mM $CoCl_2$in tank water (w/ Ca^{++})	ns	0.9988
0.1mM $CoCl_2$in Ca^{++}-free water vs. 63µM Gentamicin	ns	> 0.9999
Ca^{++} - free water (Control) vs. Tank water (w/ Ca^{++}, control)	ns	> 0.9999
Ca^{++} - free water (Control) vs. Tank water (Gentamicin control)	*	< 0.0001
0.1 mM $CoCl_2$in tank water (w/ Ca^{++}) vs. Tank water (w/ Ca^{++}, control)	*	< 0.0001
0.1 mM $CoCl_2$in tank water (w/ Ca^{++}) vs. 63µM Gentamicin	ns	0.9983
Tank water (w/ Ca^{++}, control) vs. Tank water (Gentamicin control)	*	< 0.0001
63µM Gentamicin vs. Tank water (Gentamicin control)	*	< 0.0001
Day 2		
0.1mM $CoCl_2$in Ca^{++}-free water vs. Ca^{++} - free water (Control)	*	< 0.0001
0.1mM $CoCl_2$in Ca^{++}-free water vs. 0.1 mM $CoCl_2$in tank water (w/ Ca^{++})	ns	0.1137
0.1mM $CoCl_2$in Ca^{++}-free water vs. 63µM Gentamicin	*	< 0.0001
Ca^{++} - free water (Control) vs. Tank water (w/ Ca^{++}, control)	ns	0.9983
Ca^{++} - free water (Control) vs. Tank water (Gentamicin control)	ns	> 0.9999
0.1 mM $CoCl_2$in tank water (w/ Ca^{++}) vs. Tank water (w/ Ca^{++}, control)	*	< 0.0001
0.1 mM $CoCl_2$in tank water (w/ Ca^{++}) vs. 63µM Gentamicin	ns	0.1137
Tank water (w/ Ca^{++}, control) vs. Tank water (Gentamicin control)	ns	0.9983
63µM Gentamicin vs. Tank water (Gentamicin control)	*	< 0.0001
Day 4		
0.1mM $CoCl_2$in Ca^{++}-free water vs. Ca^{++} - free water (Control)	*	< 0.0001
0.1mM $CoCl_2$in Ca^{++}-free water vs. 0.1 mM $CoCl_2$in tank water (w/ Ca^{++})	ns	0.9585
0.1mM $CoCl_2$in Ca^{++}-free water vs. 63µM Gentamicin	*	0.0004
Ca^{++} - free water (Control) vs. Tank water (w/ Ca^{++}, control)	ns	> 0.9999
Ca^{++} - free water (Control) vs. Tank water (Gentamicin control)	ns	0.8009
0.1 mM $CoCl_2$in tank water (w/ Ca^{++}) vs. Tank water (w/ Ca^{++}, control)	*	< 0.0001
0.1 mM $CoCl_2$in tank water (w/ Ca^{++}) vs. 63µM Gentamicin	*	< 0.0001
Tank water (w/ Ca^{++}, control) vs. Tank water (Gentamicin control)	ns	0.8009
63µM Gentamicin vs. Tank water (Gentamicin control)	ns	0.9983
Day 7		
0.1mM $CoCl_2$in Ca^{++}-free water vs. Ca^{++} - free water (Control)	ns	0.5360
0.1mM $CoCl_2$in Ca^{++}-free water vs. 0.1 mM $CoCl_2$in tank water (w/ Ca^{++})	ns	0.9585
0.1mM $CoCl_2$in Ca^{++}-free water vs. 63µM Gentamicin	ns	0.8009
Ca^{++} - free water (Control) vs. Tank water (w/ Ca^{++}, control)	ns	0.9983
Ca^{++} - free water (Control) vs. Tank water (Gentamicin control)	ns	0.5360
0.1 mM $CoCl_2$in tank water (w/ Ca^{++}) vs. Tank water (w/ Ca^{++}, control)	*	0.0368
0.1 mM $CoCl_2$in tank water (w/ Ca^{++}) vs. 63µM Gentamicin	ns	0.9983
Tank water (w/ Ca^{++}, control) vs. Tank water (Gentamicin control)	ns	0.2791
63µM Gentamicin vs. Tank water (Gentamicin control)	ns	0.8009

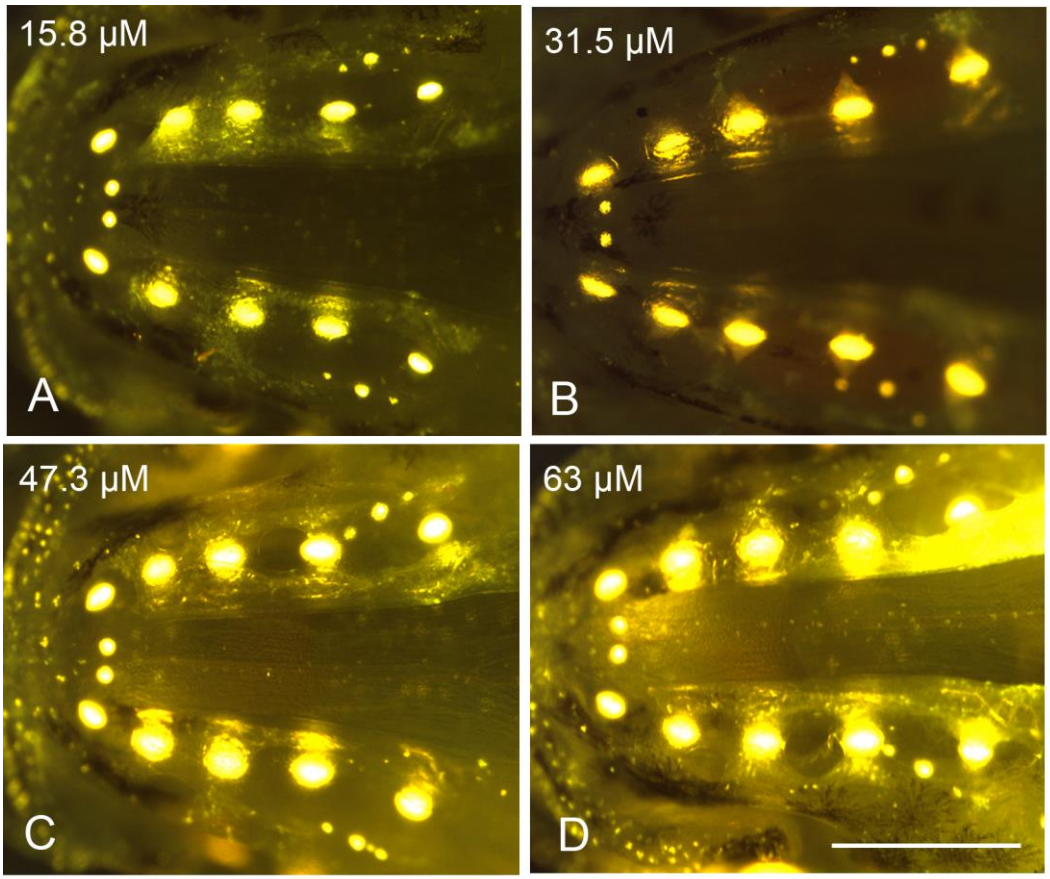

Fig. 2.1. 4-Di-2-ASP dose response in *A. stuartgranti*. A) 15.8 µM solution, B) 31.5 µM solution , C) 47.3 µM solution , and D) 63 µM solution. All images captured at the same exposure. Scale bar is ~ 1 mm.

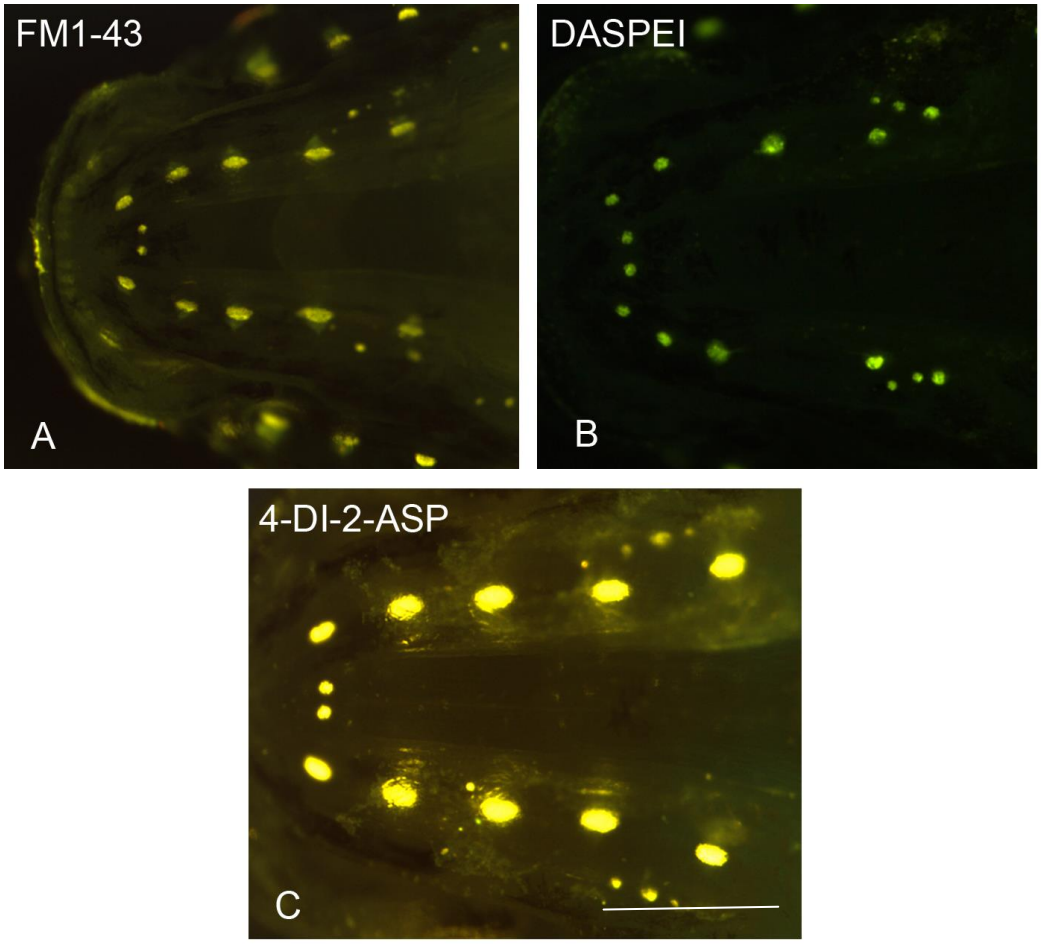

Fig. 2.2 . Ventral view of fluorescent stain comparison in mandibular canal and superficial neuromasts in *A. stuartgranti* . A) 3µM FM1-43 for 5 minutes, B) 0.01% DASPEI for 30 minutes, and C) 63µM 4-Di-2-ASP for 5 minutes. All pictures taken at the same exposure. Scale bar is ~ 1 mm.

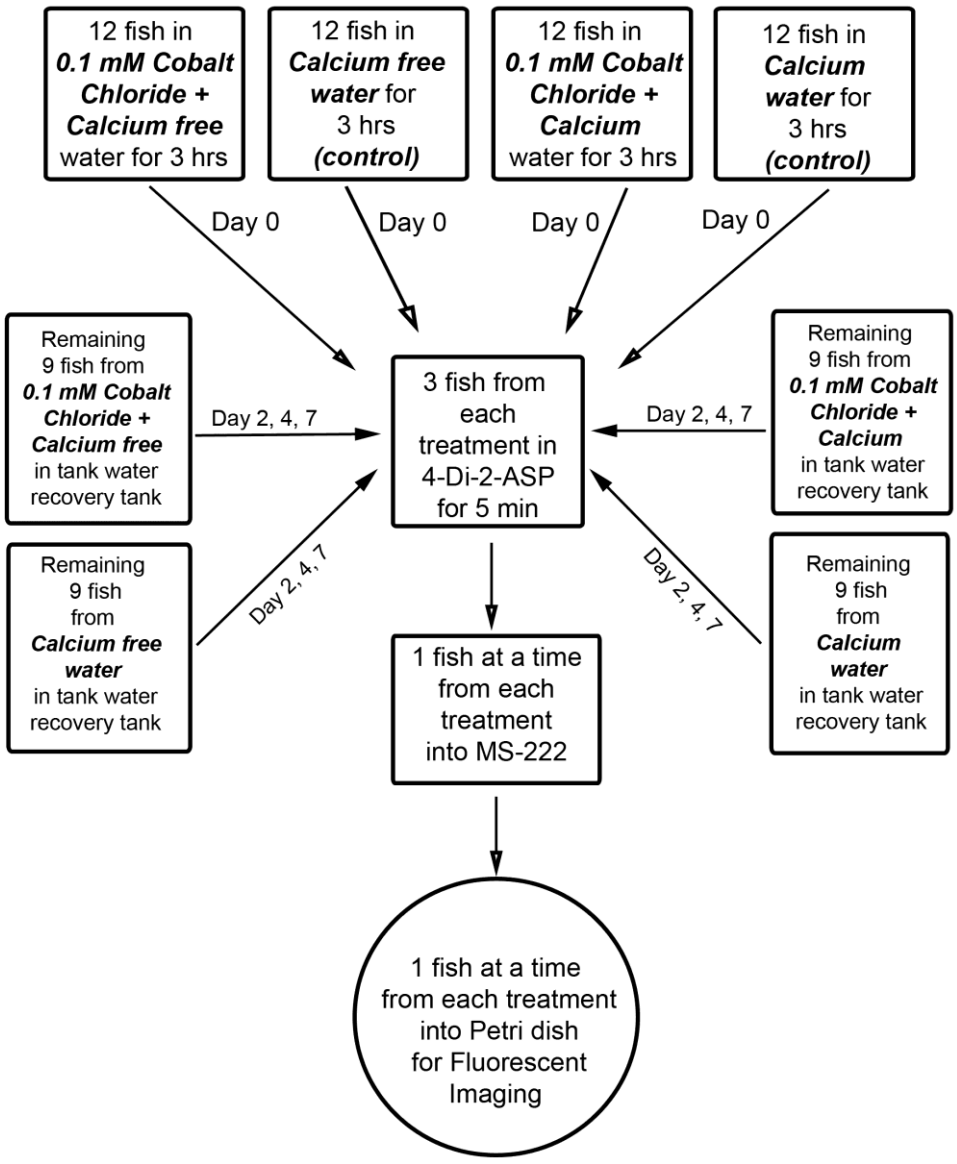

Fig.2.3. Cobalt chloride treatment flow chart for *Tramitichromis* sp. and *A. stuartgranti*. On Day 0, 3 fish from each treatment or control were stained with 4-Di-2-ASP, while the remaining 9 fish were placed in recovery tanks with tank water (w/calcium). On Days 2, 4, and 7, 3 fish were removed from the recovery tank and treated with 4-Di-2-ASP in the same way as on Day 0.

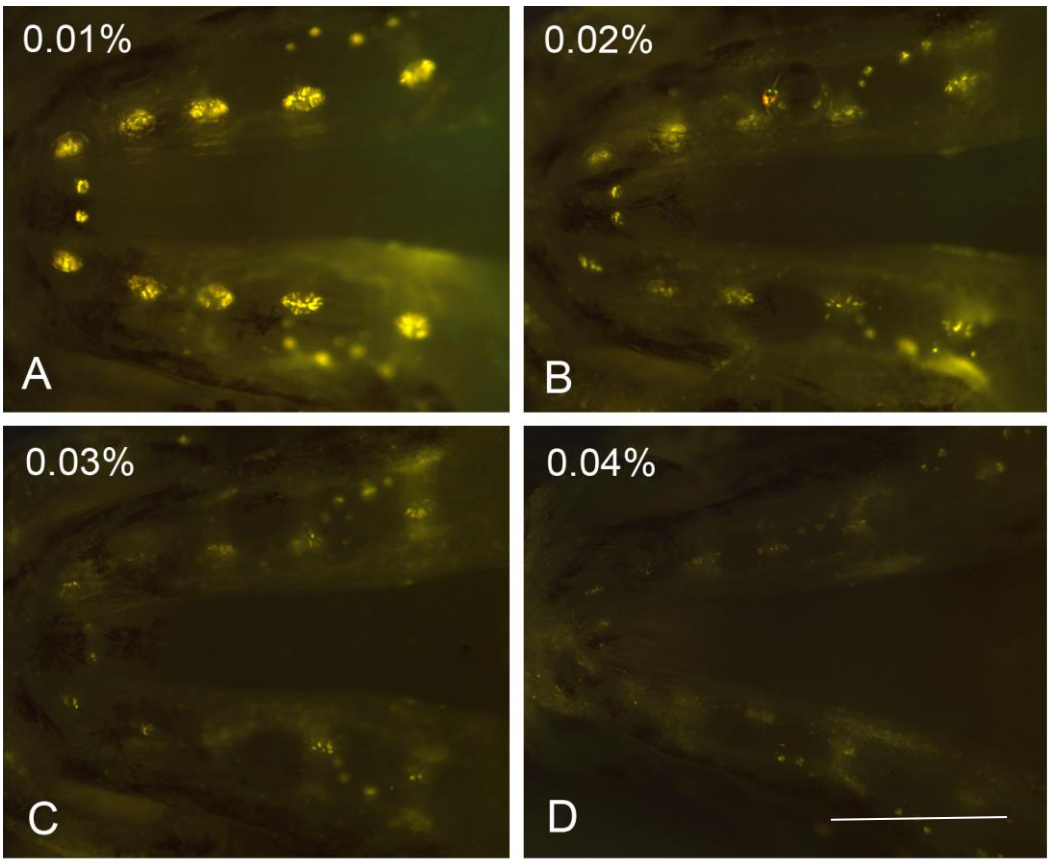

Fig. 2.4. *A. stuartgranti* gentamicin dose response. A) 0.01% gentamicin solution for 24 hours, B) 0.02 % gentamicin solution for 24 hours, C) 0.03% gentamicin for 24 hours, and D) 0.04% gentamicin solution for 24 hours. Note the diminishing fluorescence in canal and superficial neuromasts with an increase in concentration. All images taken with the same exposure time. Scale bar is ~ 1 mm.

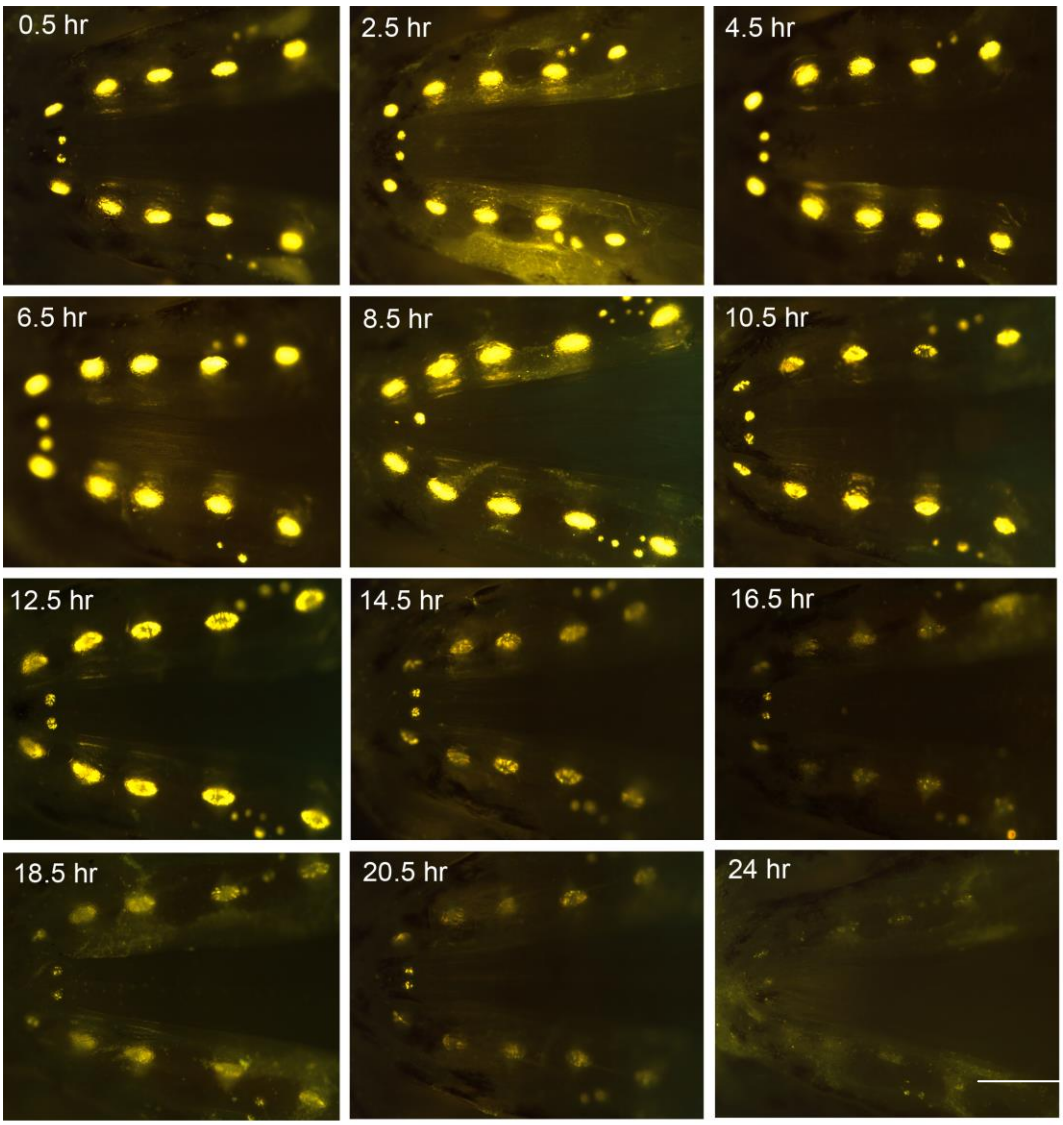

Fig. 2.5. *A. stuartgranti* 24-hour gentamicin exposure test. 0.5- 24 hours post treatment (PT) with 0.04% gentamicin solution. Note the diminishing fluorescence in canal and superficial neuromasts by 10.5 hr PT. The same exposure time was used for all images. Scale bar is ~ 1 mm.

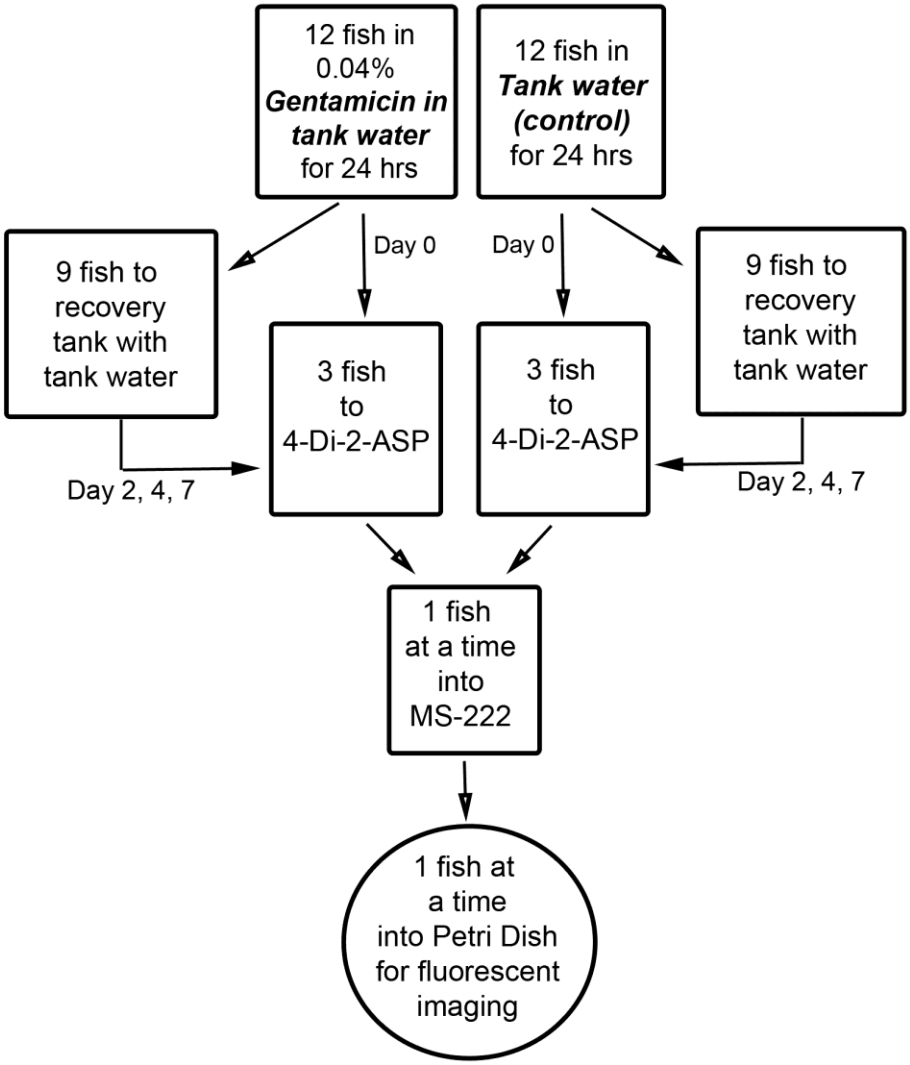

Fig. 2.6. Gentamicin treatment flow chart for *Tramitichromis* sp. and *A. stuartgranti* . On Day 0, 3 fish from each treatment were treated with 4-Di-2-ASP, while the remaining 9 fish were placed in recovery tanks with tank water (w/ calcium). On Days 2, 4, and 7, 3 fish were pulled from the recovery tank and treated with 4-Di-2-ASP in the same way as Day 0.

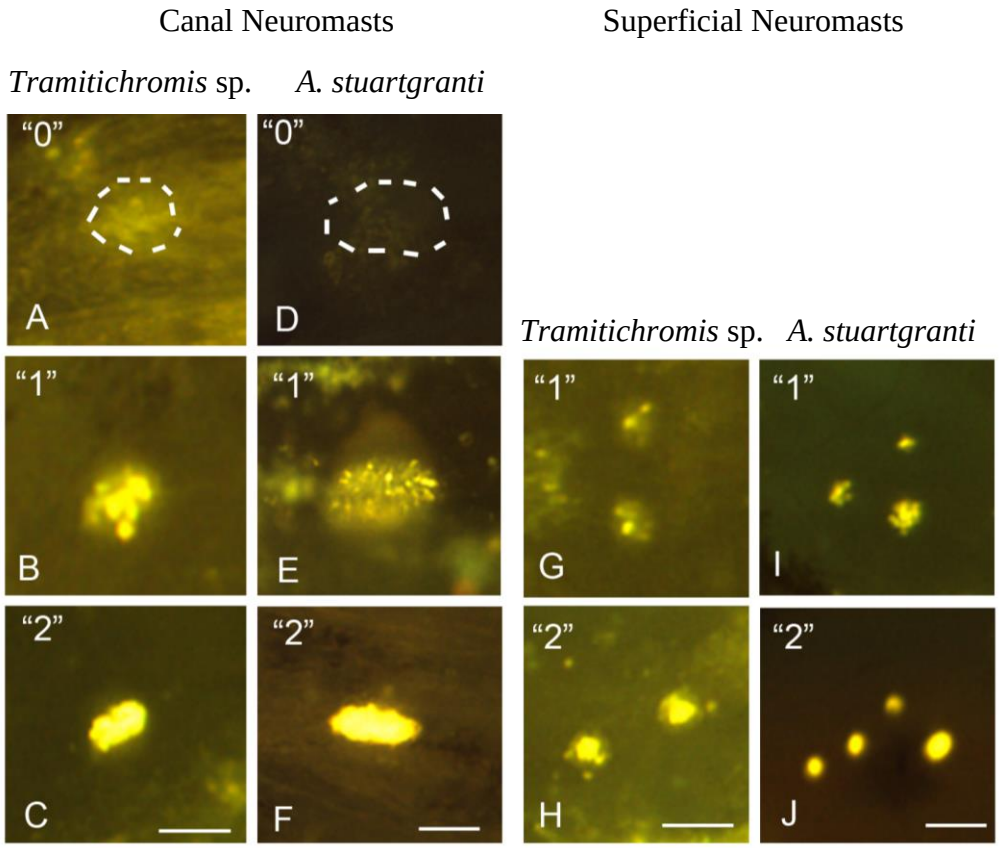

Fig. 2.7. Canal and superficial neuromast scoring in *Tramitichromis* sp. and *A. stuartgranti* . A-C) *Tramitichromis* sp. Canal Neuromasts, D-F) *A. stuartgranti* Canal Neuromasts, G-H) *Tramitichromis* sp. Superficial Neuromasts, I-J) *A. stuartgranti* Superficial Neuromasts. "0" indicates a neuromast scored as 0, "1" indicates a neuromast scored as 1, and "2" indicates a neuromast scored as 2. Note that superficial neuromasts could not be scored as 0, so those images are absent. Scale bars in C and H are ~30μm. Scale bars in F and J are ~60μm.

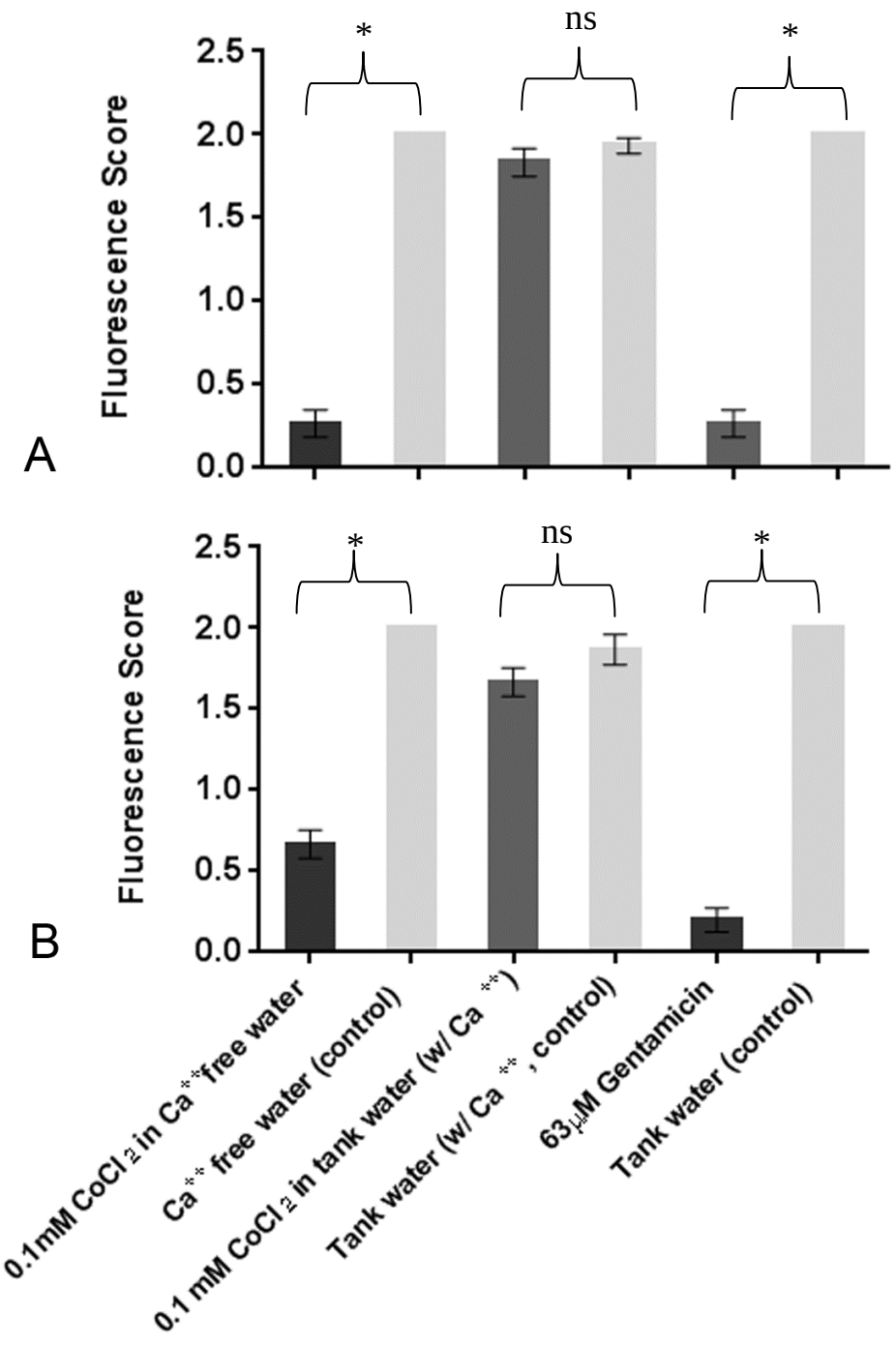

Fig. 2.8. Day 0 (day of treatment) mean canal neuromast fluorescent score in all treatments in A) *Tramitichromis* sp.and B) *A. stuartgranti.* Asterisk (*) denotes a significant difference between the treatment and control mean score. See Tables 5 and 7 for statistics.

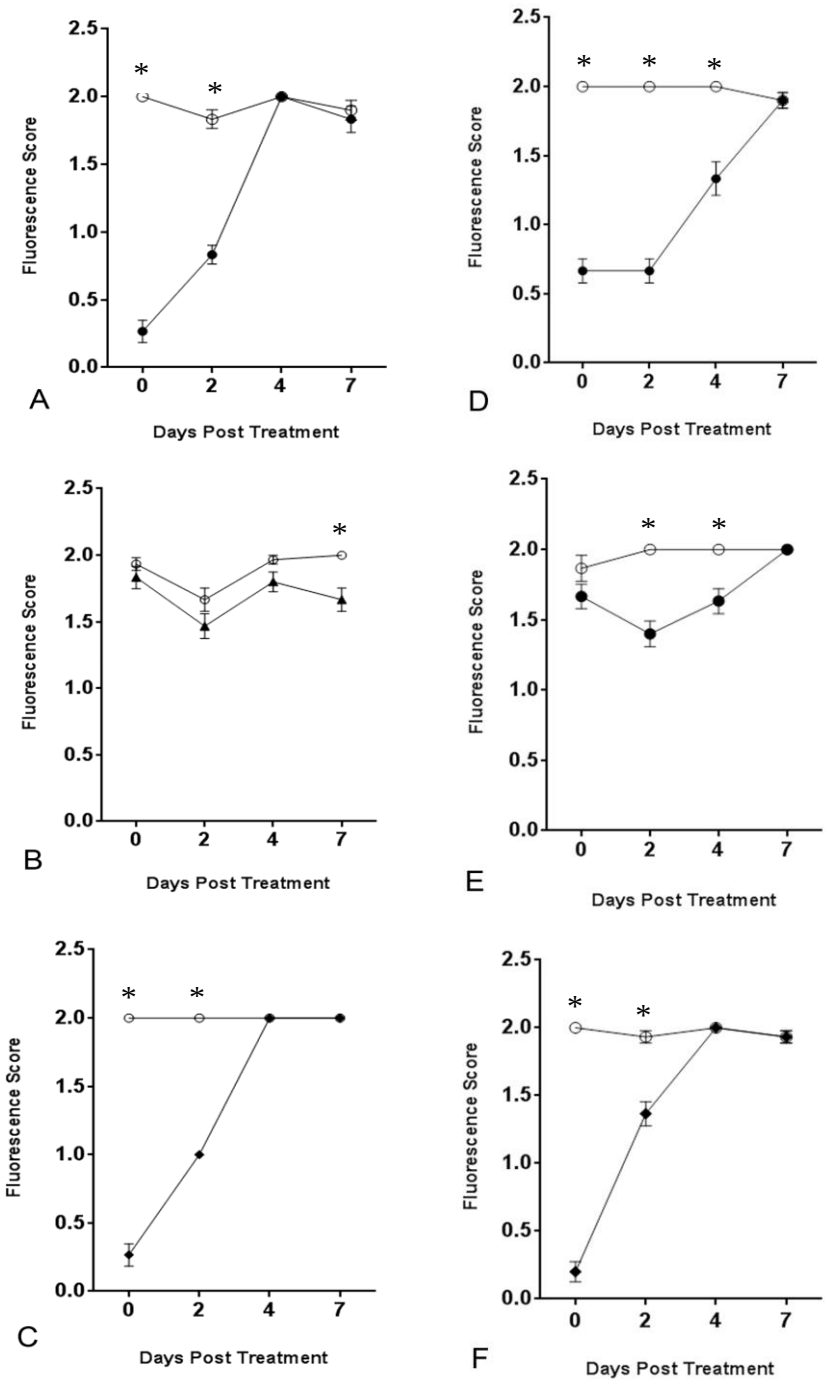

Fig. 2.9. Mean canal neuromast fluorescence score (0-2) over 7 days in all treatments. A-C) *Tramitichromis* sp. , D-F) *A. stuartgranti* ; A and D) Cobalt Chloride in Ca^{++}- free water, B and E) Cobalt Chloride in tank water (w/ calcium), C and F) Gentamicin in tank water. Asterisk (*) indicates a significant difference between the treatment and control on that day.

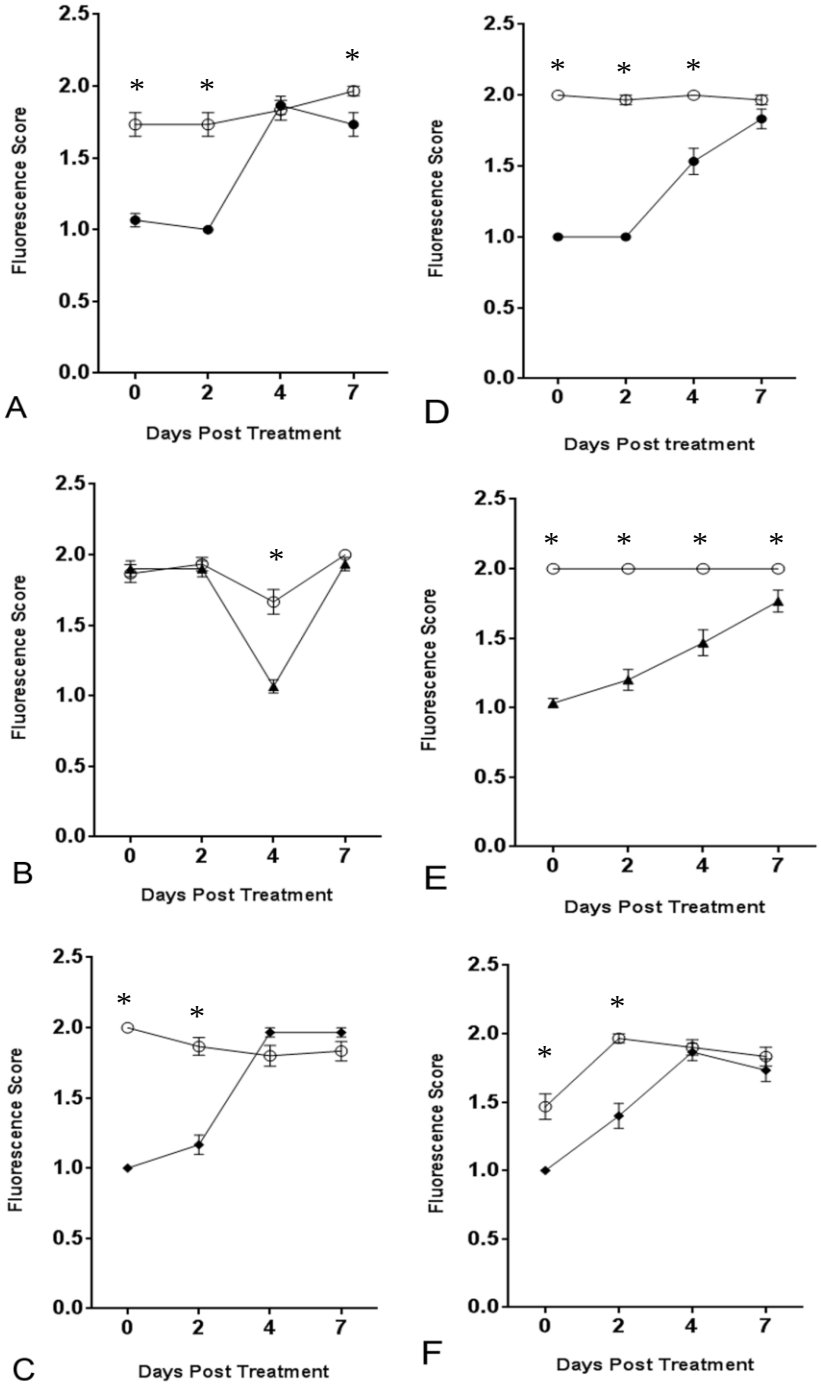

Fig. 2.10. Mean superficial neuromast fluorescence score (1-2) over 7 days in all treatments. A-C) *Tramitichromis* sp., D-F) *A. stuartgranti* ; A and D) Cobalt Chloride in Ca^{++} -free water, B and E) Cobalt Chloride in tank water (w/ calcium), C and F) Gentamicin in tank water.

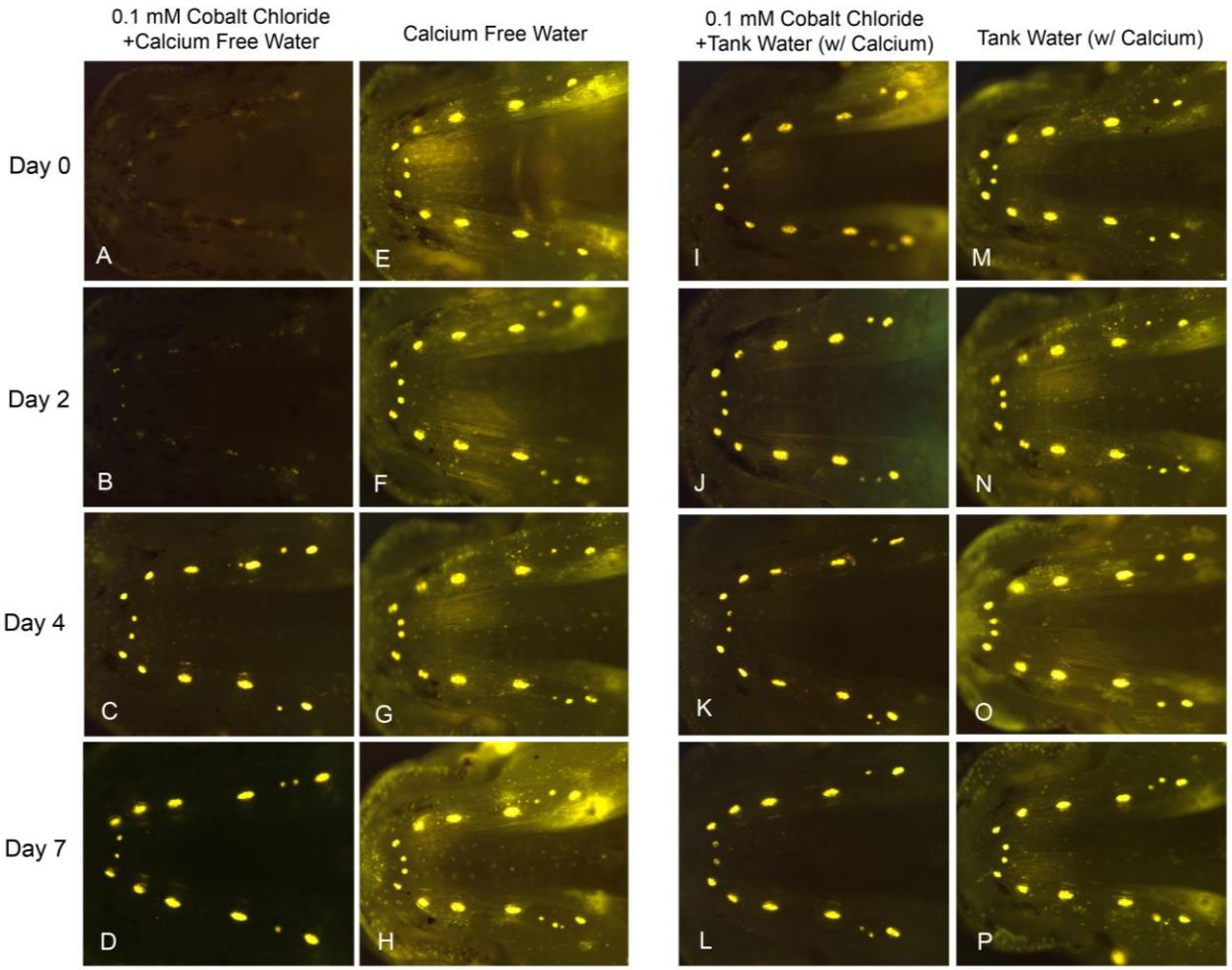

Fig. 2.11. Recovery from cobalt chloride in *Tramitichromis* sp. over 7 days as illustrated with 4-Di-2-ASP. A-D) 0.1 mM Cobalt Chloride in Ca^{++}-free water treatment for 3 hours , E-H) Ca^{++}-free water (control) treatment for 3 hours, I-L) 0.1 mM Cobalt Chloride in tank water (w/ calcium) for 3 hours, and M-P) Tank water (w/ calcium; control) for 3 hours.

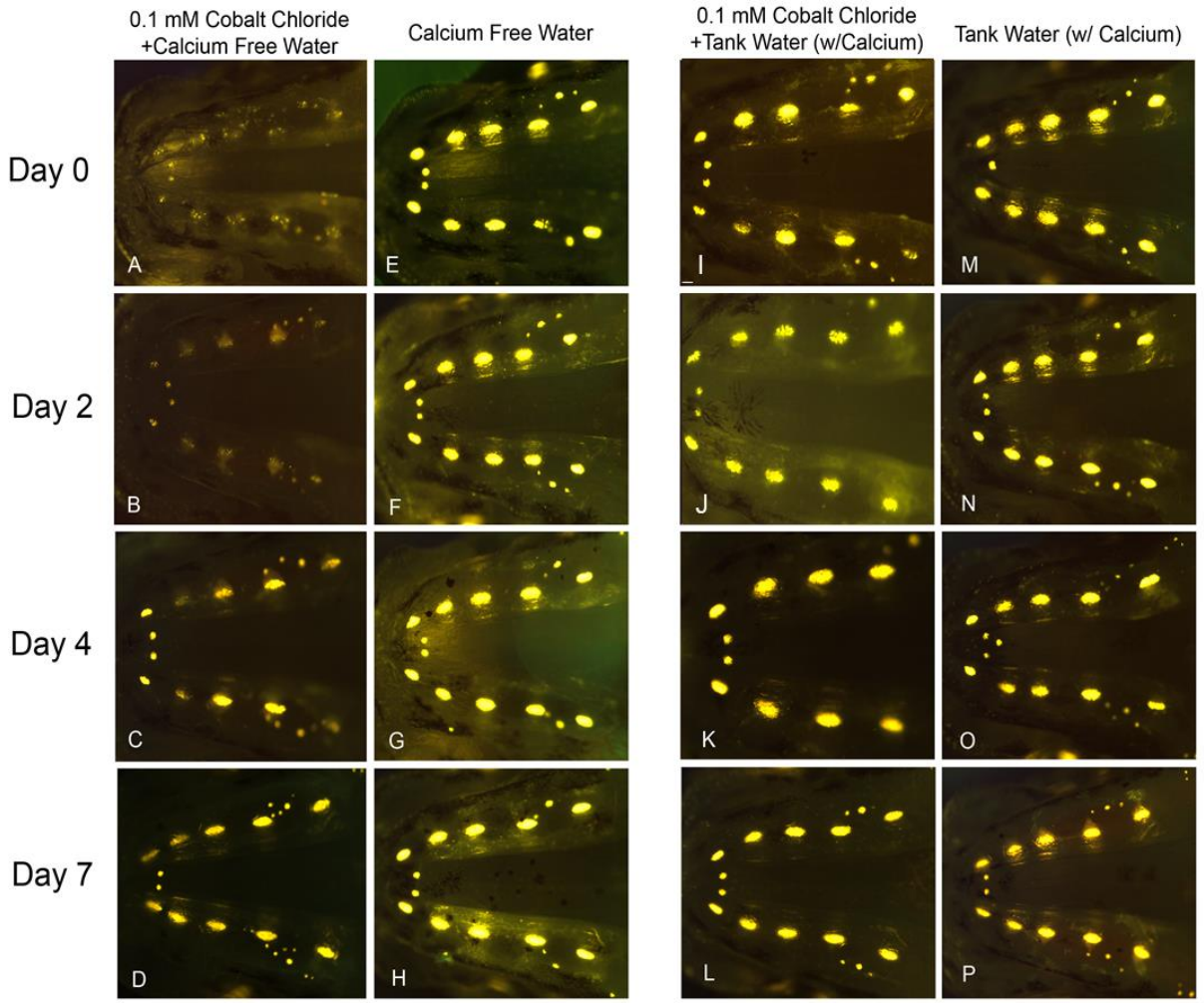

Fig. 2.12. Recovery from cobalt chloride in *A. stuartgranti* over 7 days as illustrated by 4-Di-2-ASP. A-D) 0.1 mM Cobalt Chloride in Ca^{++} -free water treatment for 3 hours , E-H) Ca^{++} -free water (control) treatment for 3 hours, I-L) 0.1 mM Cobalt Chloride in tank water (w/ calcium) for 3 hours, and M-P) Tank water (w/ calcium; control) for 3 hours.

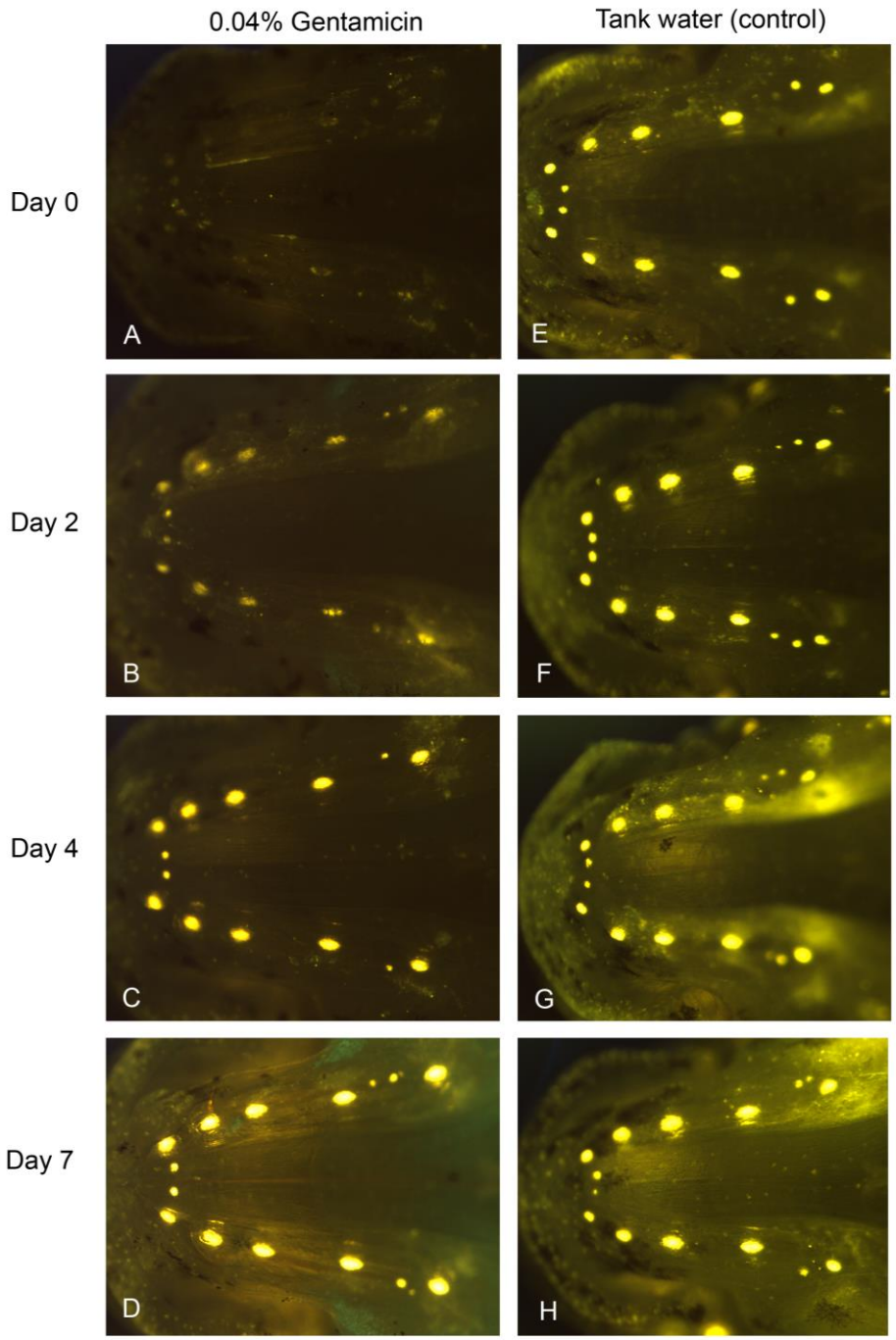

Fig.2.13. Recovery of *Tramitichromis* sp. from gentamicin over 7 days. A-D) 63 µM Gentamicin in tank water for 24 hours, E-H) Tank water (control) for 24 hours.

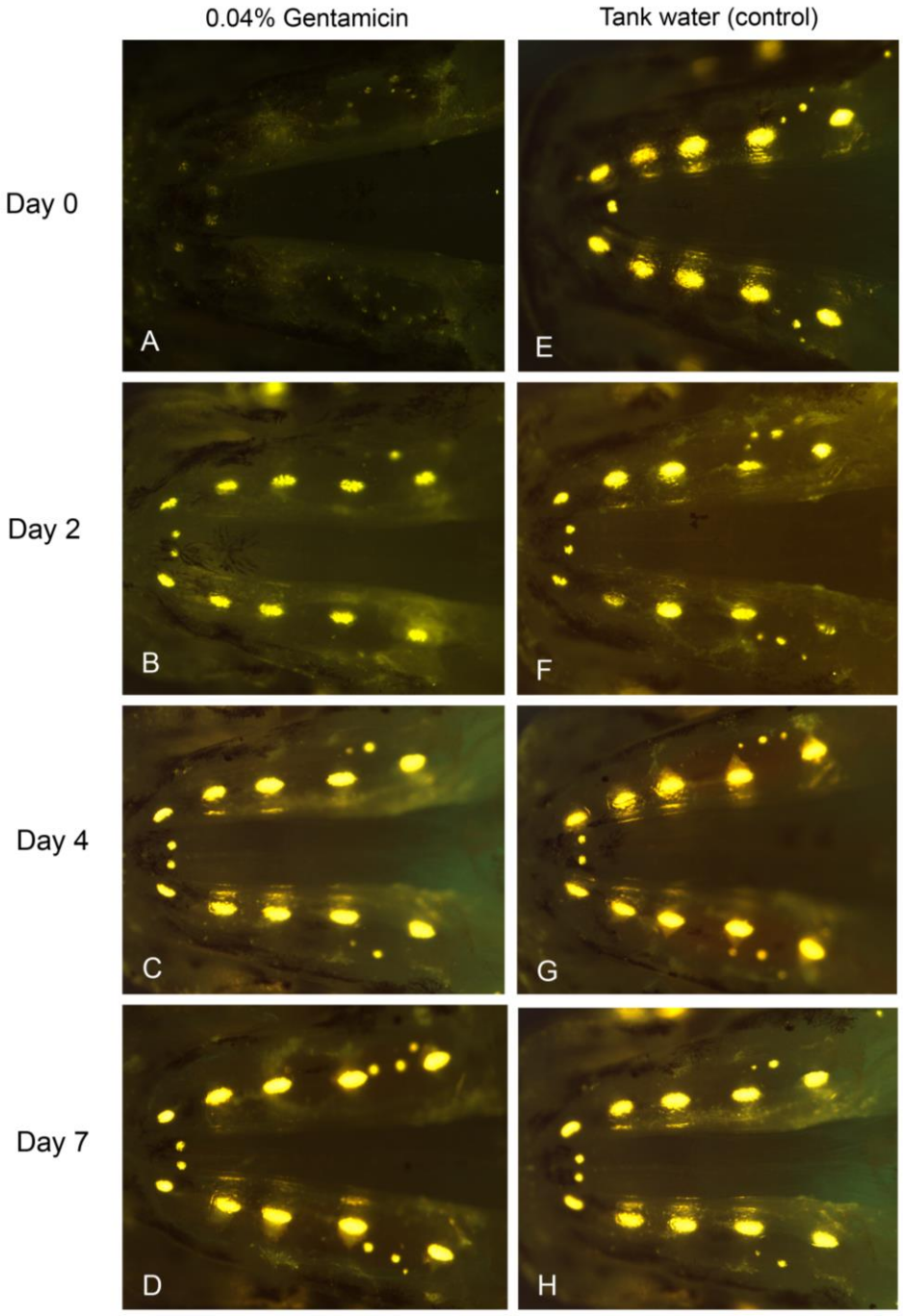

Fig. 2.14. Recovery of *A. stuartgranti* from gentamicin over 7 days. A-D) 63 µM Gentamicin in tank water for 24 hours, E-H) Tank water (control) for 24 hours.

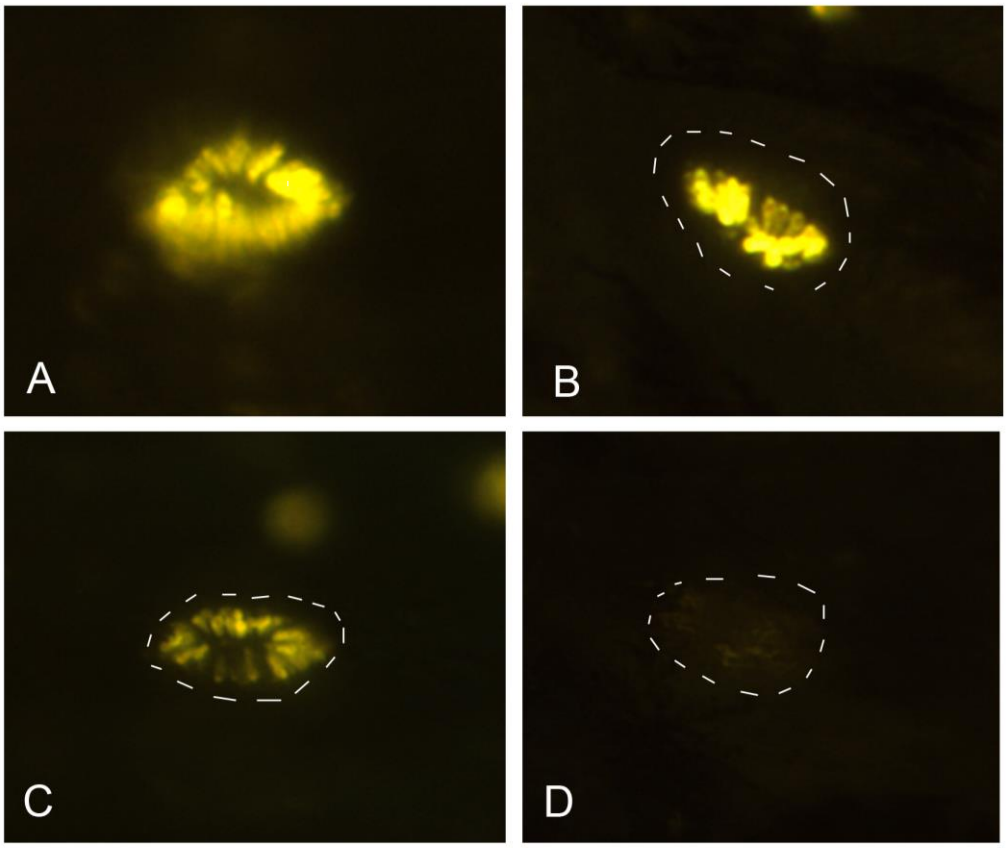

Fig. 2.15. Patterns of hair cell labeling in canal neuromasts treated with $CoCl_2$ and gentamicin in *Tramitichromis* sp. and *A. stuartgranti* . A) hair cell labeling is absent from the center of the neuromast, B) hair cell labeling is absent from the outer ring of the neuromast, C) hair cell labeling is absent around outside and center of neuromast, and D) hair cell labeling is completely absent in the neuromast. All images are representative samples from *A. stuartgranti* and *Tramitichromis* in all three treatments. SNs were too small to detect similar patterns.

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