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Acoustic Communication Modalities of the Round Goby (*Neogobius melanostomus*)

by

Patricia A Speares

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ABSTRACT

Acoustic communication is essential to survival for many fish species, however it is often understudied. This thesis investigates two areas of acoustic communication in the round goby (*Neogobius melanostomus*). These fish have no accessory hearing structures, and theory would predict they would be unable to localize sound sources, however it has been shown that round gobies can localize conspecific calls. The main concentration of this thesis is to investigate the role of the mechanosensory system in this localization ability. This thesis also includes an investigative study that makes a first attempt at examining the actual temporal characteristics of the round goby mating call. This was done by comparing the calls of males injected with Luteinizing Hormone Releasing Hormone, immediately post injection and in the weeks that followed. Inter-individual differences in call structure were also examined. This multidisciplinary approach gives an overall better view of underwater acoustic communication in this species.

STATEMENT OF AUTHORSHIP

Stacey Rowsome contributed to the work done on appendix A of this thesis. She was responsible for running but not designing the experiments explained in this appendix. Her work was done in partial fulfillment of a Honor's Thesis at the University of Windsor. She was supervised by Patricia Speares. This material is included in this thesis because it is the beginning of what will become an ongoing body of work at the University of Windsor and therefore this thesis will serve as a record of the methodology and outline of this experiment after both of these students have left the university.

DEDICATION

I dedicate this thesis to my family for all of their love and support. I especially dedicate this thesis to Chrissy, who I miss so much and who was always supportive of everything I ever wanted to do.

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First of all I would like to thank Dennis, for his constant support and understanding. Thanks for everything, from firing me when I get too confident to supplying me with an endless jar of hot sauce. You are the best advisor I could ever ask for, I feel like I have learned so much from you.

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And of course, again thank you so much to my family for all of their love and support. Thank you to Mom and Dad and Grandpa and Judy for all their support. Beth and Duck, you are the best! You guys are so successful and amazing, I am proud to be your sister. And Kevin, thanks for being probably the only one in the family that doesn't think I am crazy for wanting to work with fish for a living!

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CHAPTER I

INTRODUCTION

The Importance of Acoustic Communication

Acoustic communication is an extremely important aspect of survival for numerous organisms. Many taxonomic groups are dependent on acoustic communication to find a mate (Gerhard 1994, Ladich 2004), establish and defend territories (i.e. Myrberg and Riggio 1985, Ladich 2004) and warn others of impending dangers (Amorim 2006). Some groups such as birds have been studied for decades with regard to acoustic communication while in comparison relatively little is known about the vocal characteristics of other groups such as teleost fishes.

To make use of acoustic signals an organism must not only be able to produce sounds but they must also be able to sense and respond to them. Substantially more work has been done on the ability of fishes to sense and react to acoustic signals however, there are still gaps in our knowledge in this area. In particular much research has been done on the fish ear which is the most basic organ that allows the fish to hear (for review see Popper and Fay 1993, Fay and Popper 2000, Higgs et al. 2003). However fish have other sensory modalities that appear to both assist in the role of the ears (i.e. swim bladders and webberian ossicles) (von Frisch 1938, Schuijf and Buwalda 1975) as well as provide an alternate mechanism of detecting sound disturbances in water, completely separate from the ears (i.e. neuromasts of the mechanosensory system) (Schellart and Wubbels 1998, Coombs et al 1988). Far less is known about these complimentary structures and what role, if any, they play in actual responses by the fish such as in sound localization.

This thesis will discuss multiple aspects of acoustic communication in fish. The overall goal of this thesis is to look at both the reception and production of fish calls, in order to improve our understanding of underwater acoustic communication in general.

The Physics of Underwater Sound

To understand how aquatic organisms such as fish use acoustic communication we must first understand the properties that sound takes on under water, as acoustic properties can vary from that of sound in the air.

As sound travels in water it has two main components; particle motion (a vector component) and displacement (a scalar component). When an organism is close to the sound source it is said to be in the 'near field'. Both displacement and particle motion can be easily detected within the near field. However, as the sound moves away from its source, the particle motion component of the sound falls off much faster than the pressure component (Braun and Coombs 2000). The 'near-field/far-field' boundary is dependent upon the frequency of the sound and it is defined as the distance where particle motion is equal to the displacement. This can be mathematically found with the formula

$$\text{Boundary} = \lambda/2\Pi$$

(Rogers and Cox 1988). For example a monopole sound with a frequency of 100 Hz has a wavelength (λ) of 1487.257 cm and therefore the 'nearfield/farfield' boundary is 236.8244 cm from the sound source. It is important to understand that the far field is 'dominated by the pressure component' however the particle motion component of sound is still present but often at level that is undetectable (Mann 2006). This distinction is important because as will be discussed later, fish ears can only detect the particle motion component of sound. In a natural setting however, it would be beneficial for fish to be able to detect sounds, well beyond the near-field boundary, therefore they must rely on other mechanisms.

Fish Hearing

The fish ear consists of three semi-circular canals and three end-organs-the saccule, the lagena and the utricle (Figure 1). The function of the canals and the utricle are mainly vestibular while the lagena and the saccule are considered to be auditory in most fish (Popper et al 1982). However, there are exceptions to this such as fish in the family Clupeidae which use the utricle as their primary hearing organ (Blaxter et al. 1981, Mann et al 2001). All of the end-organs contain a sensory epithelium (or macula), which is made up of innervated sensory hair cells connected to supporting cells. The hair cells consist of one longer true cilium (kinocilium) and many smaller stereocilia (Popper et al 1988). This sensory epithelium is connected to or very closely overlain by a dense calcareous otolith (Figure 2). When a disturbance, such as a sound wave, hits the fish, the otolith is displaced relative to the epithelium causing a shearing effect. This bending of the hair cells of the epithelia causes an action potential to be sent to the brain. The maximum depolarization of the hair cells occurs when the hair cells are bent towards the kinocilium and the maximum repolarization occurs when the stereocilia bend in the opposite direction (Hudspeth and Corey 1977).

Although most fish ears share the characteristics described above there is a great deal of interspecific variation in fish ears (Popper and Coombs 1982). One area of variation is in

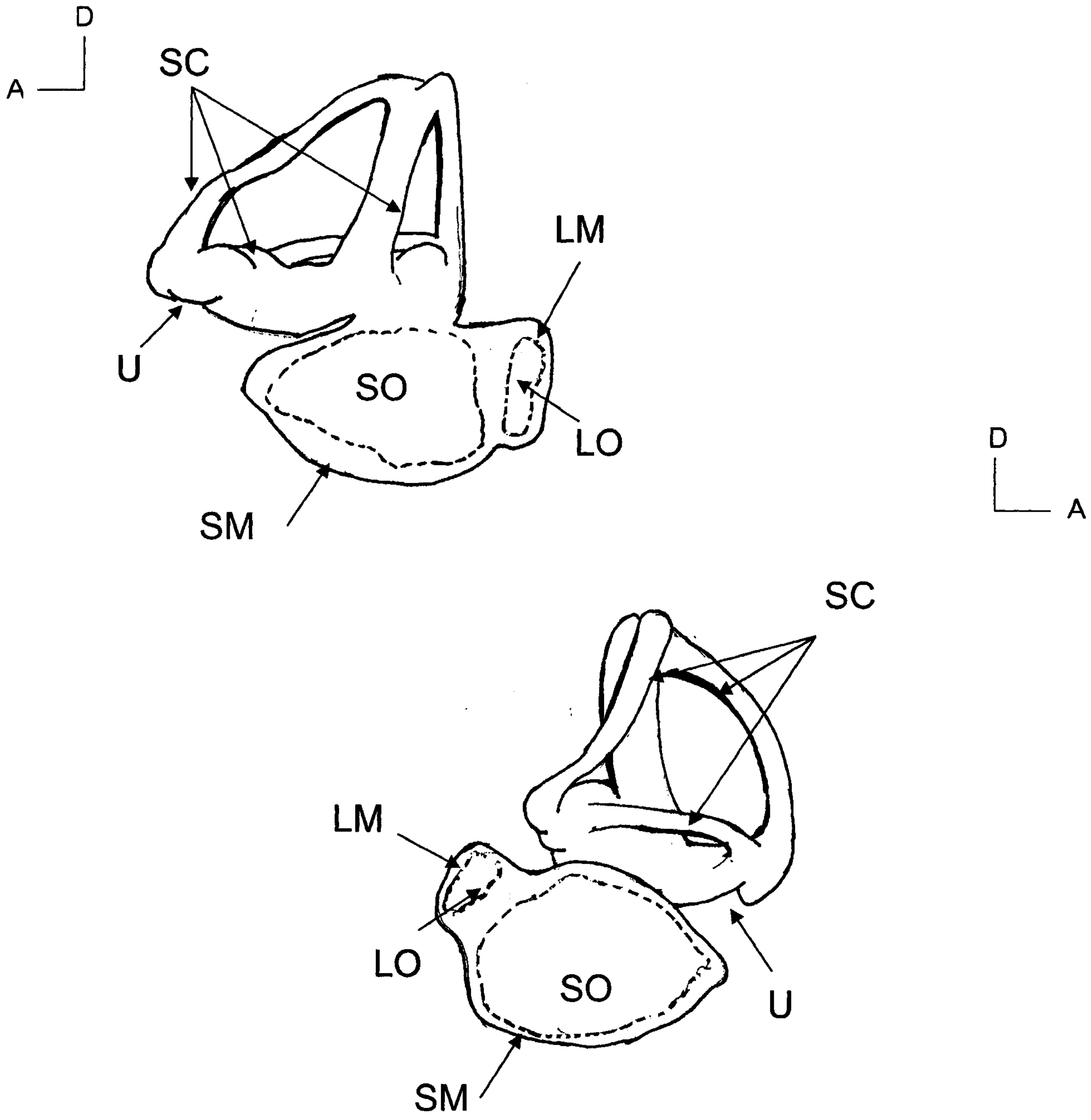


Figure 1: The basic ear morphology of a fish; modified from original image copyright: Dr. Arthur N. Popper, Laboratory of Aquatic Bioacoustics, University of Maryland. Abbreviations as follows; SC: semi-circular canals; U: utricle; SO: saccular otolith; SM: saccular macula; LO Lagena otolith; LM: lagena macula.

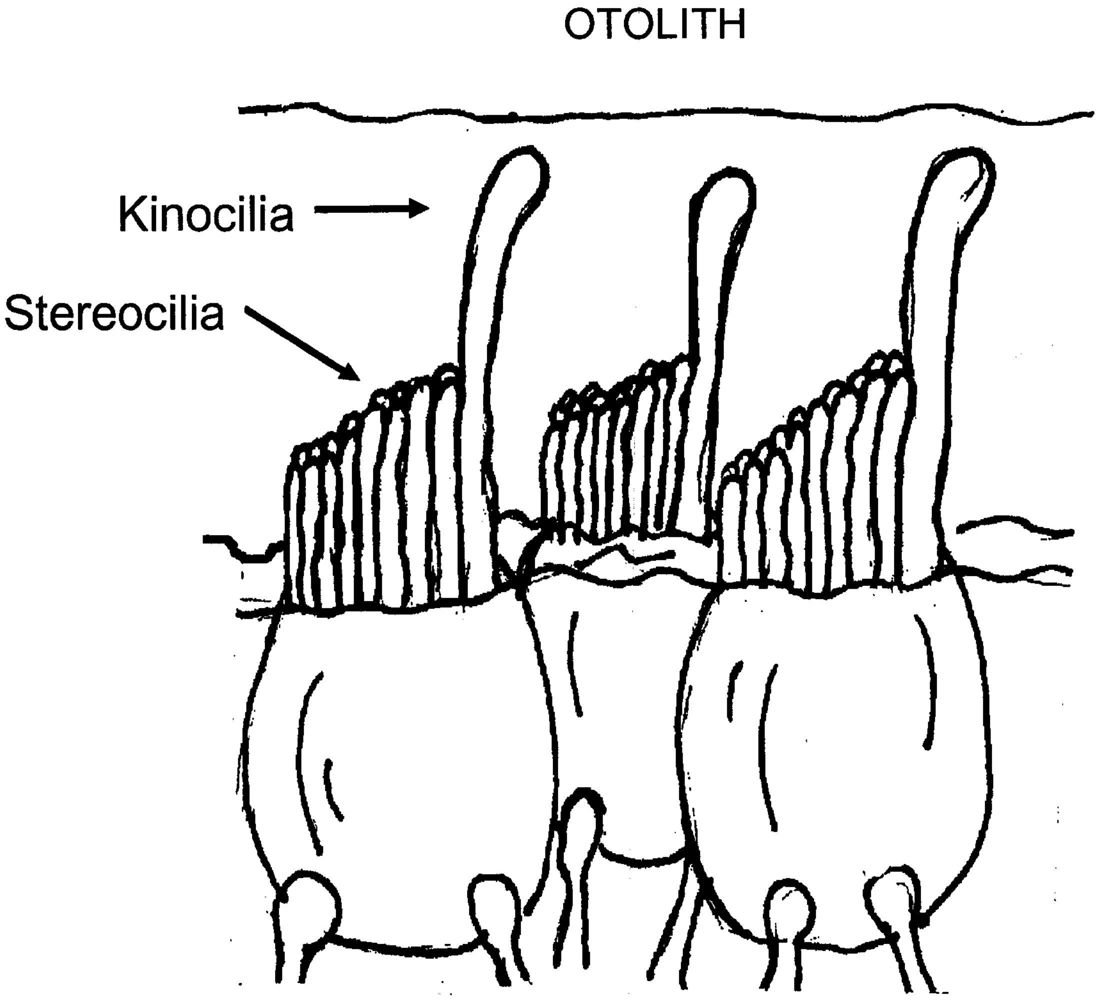
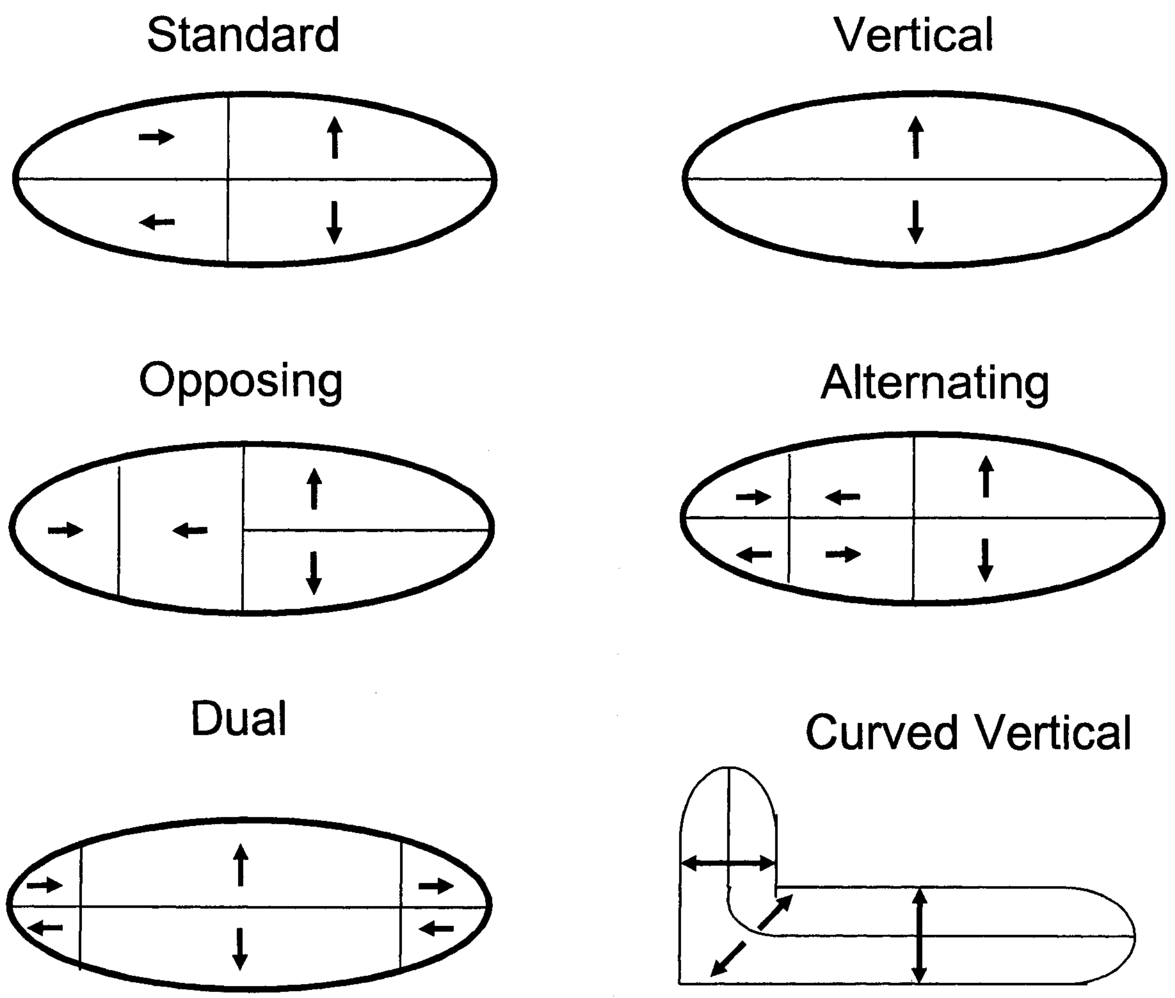


Figure 2: The sensory hair cells of the ear are shown overlain by a more dense calcalious otolith as found in the end-organs of the fish. Modified from Rollo 2005.

the polarization of the hair cells of the sensory epithelia, especially in the saccule (representative polarization patterns shown in figure 3). Polarization of hair cells is characterized by determining in which direction the kinocilia of the hair cells are pointing. Most fish have at least four quadrants of polarity (i.e. two quadrants pointing horizontally and two quadrants pointing vertically) in their saccular macula, however some fish such as those with a direct (or close) connection between the swim bladder and the ear (see Hearing Specializations Section) have fewer polarity quadrants (Popper et al. 1982).

The six otolith end-organs (3 paired organs) each have hair cells in which the kinocillium, and therefore the axis of greatest sensitivity points in various directions. So, while the otoliths may only be moving in one direction, the hair cells are all being stimulated in different directions, depending upon their polarity. This could be responsible for the ability of fish to directionalize sounds (Rogers and Cox 1988). However, there is one main problem with this argument. If directionality was determined by the end-organs of the ears alone, then sounds originating from completely opposite directions (180° from each other) would stimulate the ears in exactly the same way (Schuijf and Buwalda 1975). This '180° ambiguity problem' is a critical issue because it would mean that a fish would only have a fifty percent chance of orienting in the correct direction (and a fifty percent chance of facing the complete opposite direction). This of course, is not how fish have been observed to behave; therefore this polarization theory may not be the complete picture. The answer to the 180° orientation problem is thought to be found in hearing specialization structures such as swim bladders and other accessory



Standard

Vertical

Opposing

Alternating

Dual

Curved Vertical

Figure 3: A schematic representation of typical patterns of polarization in observed in the saccule of the fish ear. The arrows represent the direction of the kinocillium of the hair cells in the respective regions. Modified from Popper and Fay 1999.

structures (See next section). These structures are sensitive to the displacement component of sound and have been shown to be able to indirectly stimulate the end-organs in the ears. The current theory is that air-filled structures move and vibrate when stimulated by a disturbance such as a sound wave in the water (von Frisch 1938). This vibration indirectly stimulates the end-organs of the ear by thus transforming the pressure component of sound into particle motion. This simultaneous detection and analysis of both the particle motion and pressure component of sound could be sufficient for directional analysis (Schuijf and Buwalda 1975, Schuijf and Buwalda, 1980).

Specializations/ Sensitivities

Many fishes use accessory structures which are gas-filled sacs, in coordination with their ears, (Figure 4) to improve overall hearing sensitivities. Fish without any air-filled structures, or those that have air-filled structures far removed from the ear are commonly referred to as hearing generalist fish. These hearing generalist fish are known to be able to hear sounds less than 1000 Hz at generally high sound intensities (Amoser and Ladich 2005). Hearing specializations can be as basic as a simple swim bladder however, fish with more complicated specializations are able to hear a wider range of sounds. The specializations include gas filled extensions of the swim bladder that can come close to the ears as in the Sciaenidae (Ramcharitar et al. 2006) or the Holocentridae (Popper and Coombs 1979) or that actually touch the ear as in fish from the order Clupeiformes (Mann et al 2001). These groups have much higher sensitivities than that of hearing generalists. Holocentrids can hear up to 3kHz while sciaenids and clupeiforms are sensitive up to 4kHz, with one subfamily within clupeiforms (the Alosinae) able to detect

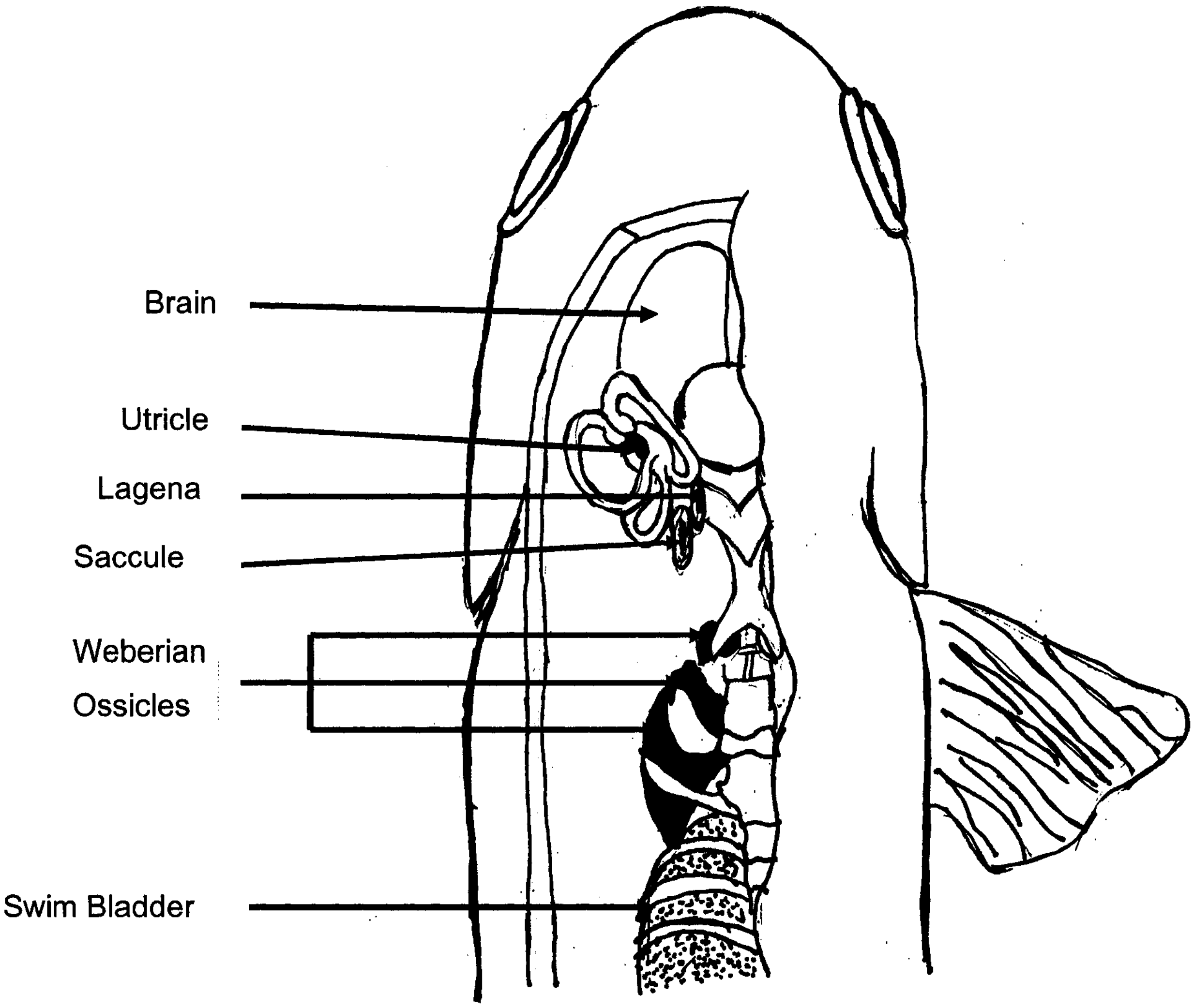


Figure 4: The arrangement of the ears and accessory hearing structures in a generalized fish. Weberian ossicles (shaded in black) and swim bladder (dotted) not present in all fish. Modified from von Frisch 1938.

ultrasound, up to 180 kHz (Mann et al. 1997, Mann et al. 2001). Another type of specialization is modified vertebra which connect (or nearly connect to) the swim bladder to the ear. These vertebrae can be found in the superorder Ostariophysi, where they work to transfer vibrations of the swim bladder to the otoliths of the ears allowing the fish in this family to be able to detect sound up to 4 kHz (Popper and Fay 1999).

The loss of a swim bladder and the development of various hearing specializations is widespread throughout teleosts (Figure 5), suggesting that these structures evolved many times independently (McCune and Carson 2004). There are many theories as to why these specializations may have evolved. For example goldfish (*Carassius auratus auratus*) are not known to be vocal however, being Ostariophysii, they have very sensitive hearing up to several kHz so vocal abilities may not an accurate way to predict sensitivities. Clupeids are sensitive to ultrasound (Mann et al 1997) but not known to produce them, however it might be beneficial to hear ultrasonic sounds of predators such as dolphins (Mann et al. 1998) (In 2004 Wilson et al, found that one species of clupeids has been shown to produce ultrasonic frequencies when they expel air but this seems to be a passive sound). The hearing abilities of a fish may be shaped by interactions with its environment and may also may be somehow correlated with the amount of ambient noise in their environment (Amoser and Ladich 2005, Fay and Popper 2000).

The Mechanosensory System

An alternate mechanism for sound reception in fish is the lateral line system. This system is composed of end-organs collectively known as neuromasts. These contain mechanoreceptive hair cells, connected to basal and supporting cells and are found

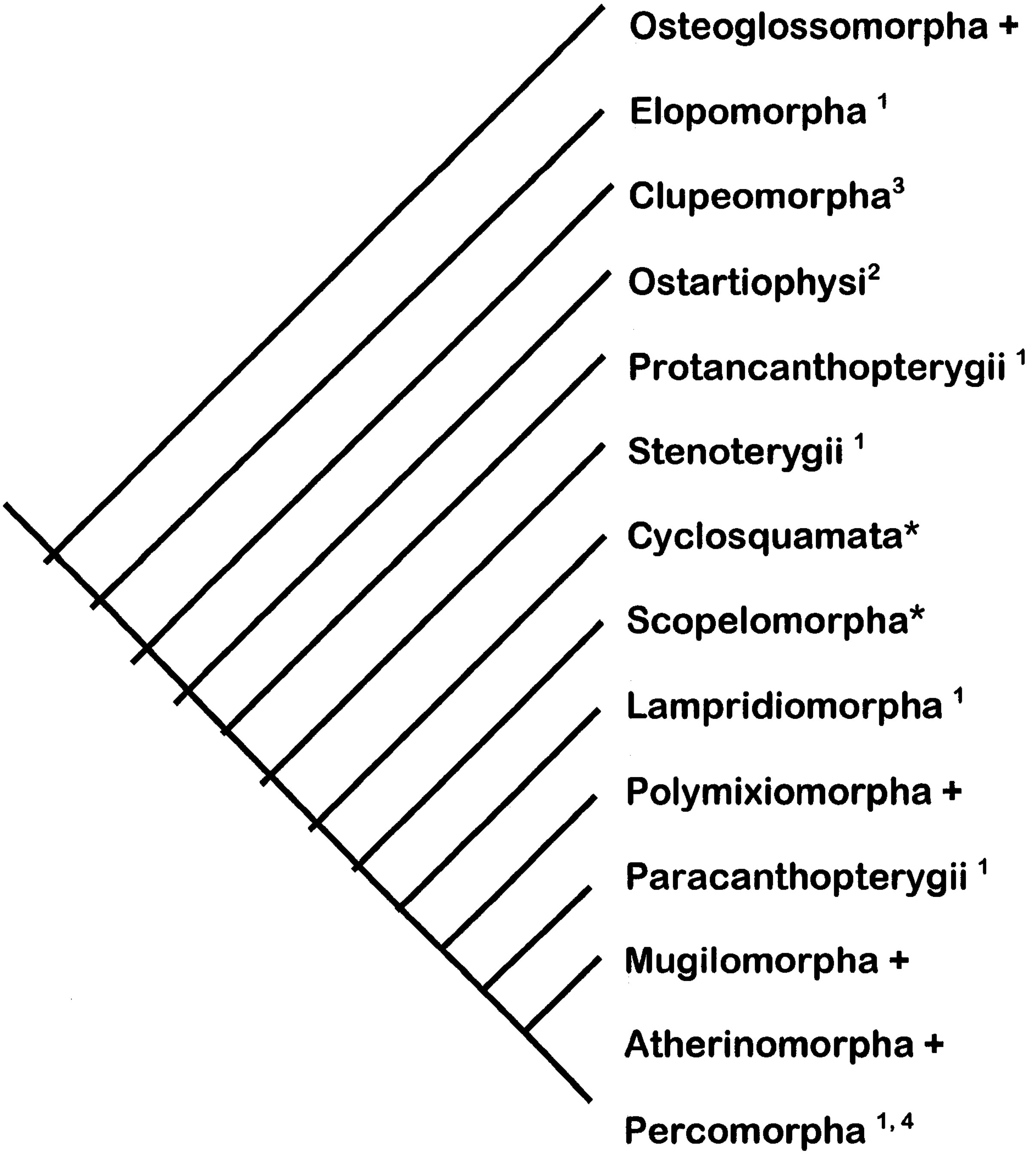


Figure 5: The distribution of hearing specializations throughout the superorders of teleosts. *:all members of superorder lack a swim bladder; +: all members of this superorder have swimbladders; 1: Swim bladder trait has disappeared at least once in this superorder; 2: Weberian ossicles present in some fish within this superorder; 3:Auditory bullae; 4:specialized swim bladder extentions within some fish of this superorder (Classifications from McCune and Carlson 2004

throughout the head and the body of the fish (Figure 6). Like the ears of the fish, these end-organs are displacement sensitive, but instead of having an otolith these structures are covered by a more dense gelatanous cupula that is displaced relative to the hair cells once again causing an action potential (Figure 7).

The sensitivity of the neuromasts is generally 200 Hz and lower, slightly overlapping that of the ears (Braun and Coombs 2000). In addition to sound reception this sensory system has been found to be responsible for many behavioral functions including schooling (Partridge and Pitcher 1980), rheotaxis (Montgomery et al 1997, Baker and Montgomery 1999) and detection of prey (Montgomery and Macdonald 1987).

There are two main categories of neuromasts (Table 1). Superficial neuromasts are more sensitive to water currents (Coombs and Montgomery 1994, Montgomery et al. 1994, Chagnaud et al. 2007) and found on the surface of the fish's head and body. Canal neuromasts are more sensitive to changes in acceleration and are found in shallow boney, fluid-filled canals throughout the head and body of the fish (Montgomery et al. 1994, Coombs and Janssen 1990, Kroese and Schellart 1992). Morphologically these two types of neuromasts seem to be similar but not identical. It has been shown that the canal neuromasts are larger and can be composed of over 100 hair cells while the superficial neuromasts are smaller and may only contain about 40 hair cells (Song et al. 1995). Also, while both have a gellatanous cupula that covers the kinocillium and the sterocilia, the length of the kinocilium and the density of hair cells was shown to be greater in the superficial neuromasts (Song et. al 1995). There is also a large degree of variation as to the type, position and density of both kinds of neuromasts depending on the species.

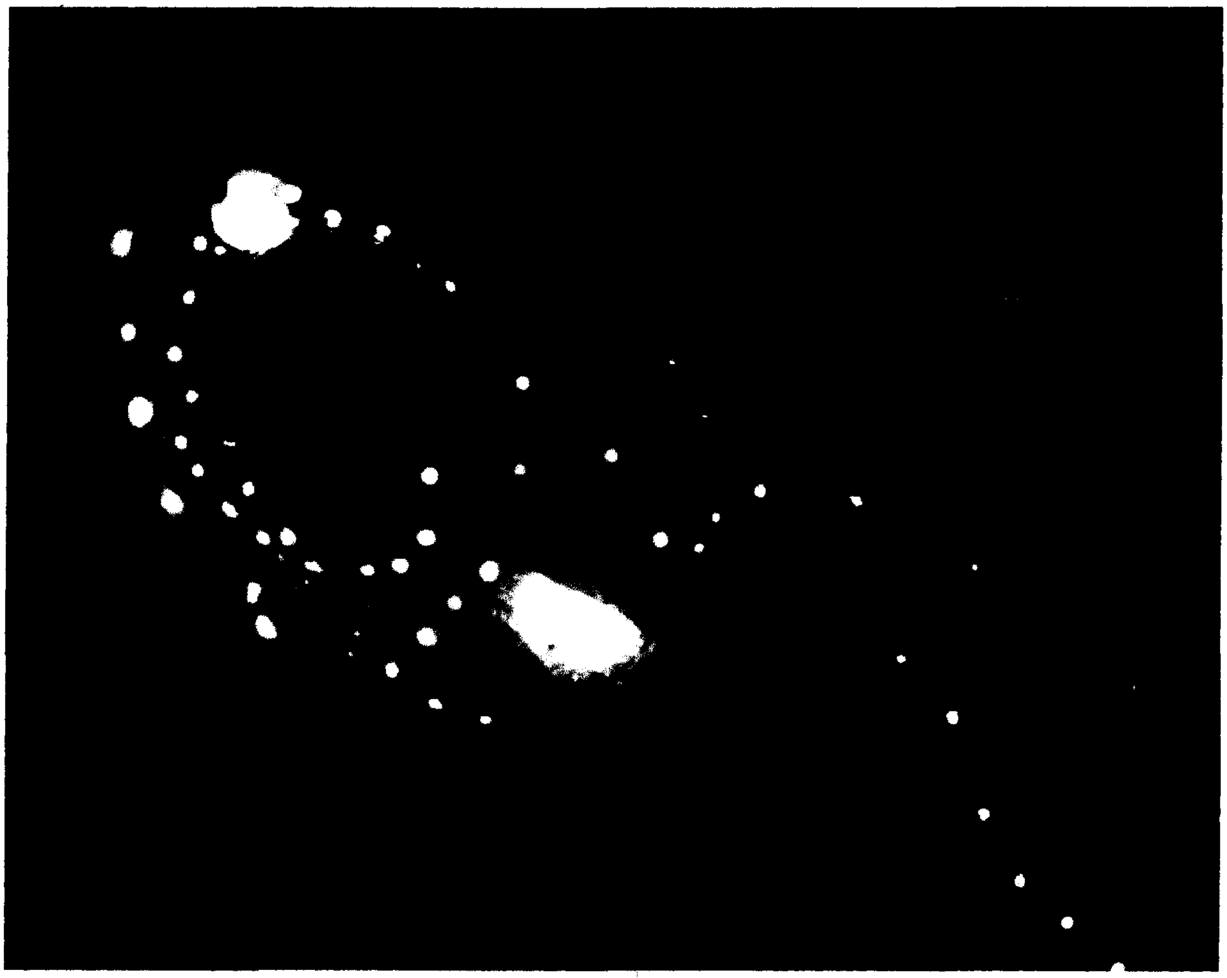


Figure 6: The superficial neuromasts (depicted as white dots) on a larval zebra fish (*Danio rerio*). This fish is stained with the fluorescent dye DASPEI. Picture taken by Eva Jawoski.

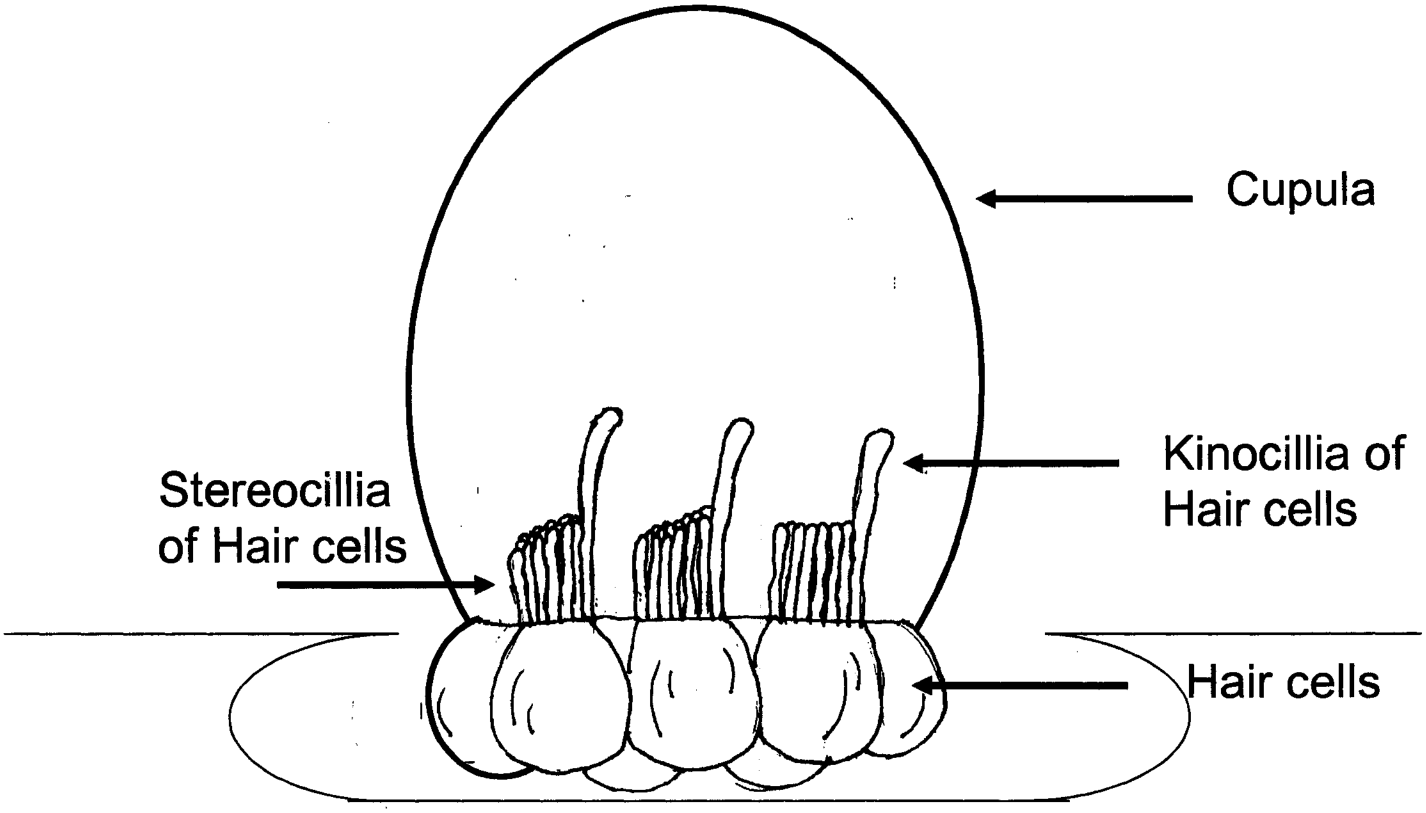


Figure 7: A pictorial representation of a neuromasts, the functional unit of the fish mechanosensory system. A sound wave or other disturbance will cause relative movement of the cupula, causing a bending of the hair cells, allowing for an action potential to occur.

Table 1: A comparison of canal and superficial neuromasts (Information from Song et al. 1995, Coombs et al. 2001)

Distinguishing Characteristic	Superficial Neuromasts	Canal Neuromasts
Peak Sensitivity	Less than 30 Hz	30-150 Hz
Number of Hair cells per Neuromast	Approx. 40	Over 100
Location	Surface of skin on head and body	In fluid filled canals throughout head and body
Known Functions	Rheotaxis, Prey Detection, water currents	Orientation, Prey Detection, changes in acceleration
Aminoglycoside Antibiotic Sensitivity	Ablated by Streptomycin	Ablated by Streptomycin Ablated by Gentamycin

Rouse and Pickles (1991) investigated the lateral line system of the bullseye fish (*Parapriacanthus ransonetti*) and the cardinal fish (*Apogon cyanosoma*) and found that at any time immature neuromasts and mature neuromasts were located together, indicating a high rate of death and regrowth in these cells. A high rate of turnover, allows these neuromasts to be studied by means of surgical and chemical ablations. Common chemical ablation mechanisms are ototoxic antibiotics such as gentamycin (chemically ablates only canal neuromasts) (Song et al 1995), streptomycin (chemically ablates both superficial and canal neuromasts) (Montgomery et. al. 1997) and cobalt chloride (chemically ablates both systems, can only be used for freshwater fish) (Karlsen and Sand 1987). All of these methods are reversible only when the correct concentrations are used and not for prolonged or repeated amounts of time (Janssen 2000).

Ablation studies have brought to light the fact that these two systems can work independently or together. Canal neuromasts were found to be responsible for orientation responses to vibrating sphere, used to stimulate prey, within one body length (Coombs et al. 2001). Superficial are independently responsible for tasks such as rheotaxis (Montgomery et al. 1997, Baker and Montgomery 1999).

Fish Vocalizations

The acoustic behavior of fish is an area of research in which some work has been done but much more is needed. It is hard to make generalizations about this field because while there have been many studies that examine the ability of individual species to vocalize (for a review see Amorim 2006, Ladich 2004), few studies to date have looked at this from a group or even phylogenetic perspective (Lugli et al. 1996) (See figure 8 for a 'typical call'). This review considers only signals that are known to be non-passive and made with actual body morphology (i.e. made with muscles or bones) will be considered actual acoustic signals. Other external signals such as hitting the substrate may also be equally as important but for simplicity sake they will not be considered in this review.

Vocalizations on Reproduction

Most fish vocalizations occur in a reproductive context; this includes courtship, spawning and male-male aggressive interactions. It is mainly the males of the species that are known to be vocal. Fish in the family Gobiidae (e.g. Lugli et al. 1996, Lindström and Lugli 2000), and the family Batrachoidae (e.g. Gray and Winn 1961) and darters in the subgenus *Catonotus* (Johnston and Johnson 2000b) are known to make vocalization while guarding nests. Nest guarding males may use acoustic vocalizations to attract many females (Lugli et al 1996, Lugli et al. 2004, Lindström and Lugli 2000) or possibly to aggressively confront other males (Lugli et al 2004, Gray and Winn 1961) without having to leave their nest, thus exposing their shelters to invaders and any eggs present to predators. Vocalizations may occur alone or as part of a more complex visual display as in many species of African cichlids (Amorin et al. 2004, Ripley and Lobel 2004) which emit courtship vocalizations, along with a 'quiver' or 'circle' behavior. It was found that these behaviors began just days before the first spawning activity is observed (Ripley and Lobel 2004). The role of sound production of the Mozambique tilapia (*Oreochromis mossambicus*) in agonistic and mating interactions is observed during hierarchy formation. It was found in this species, where only territory holding males produce calls,

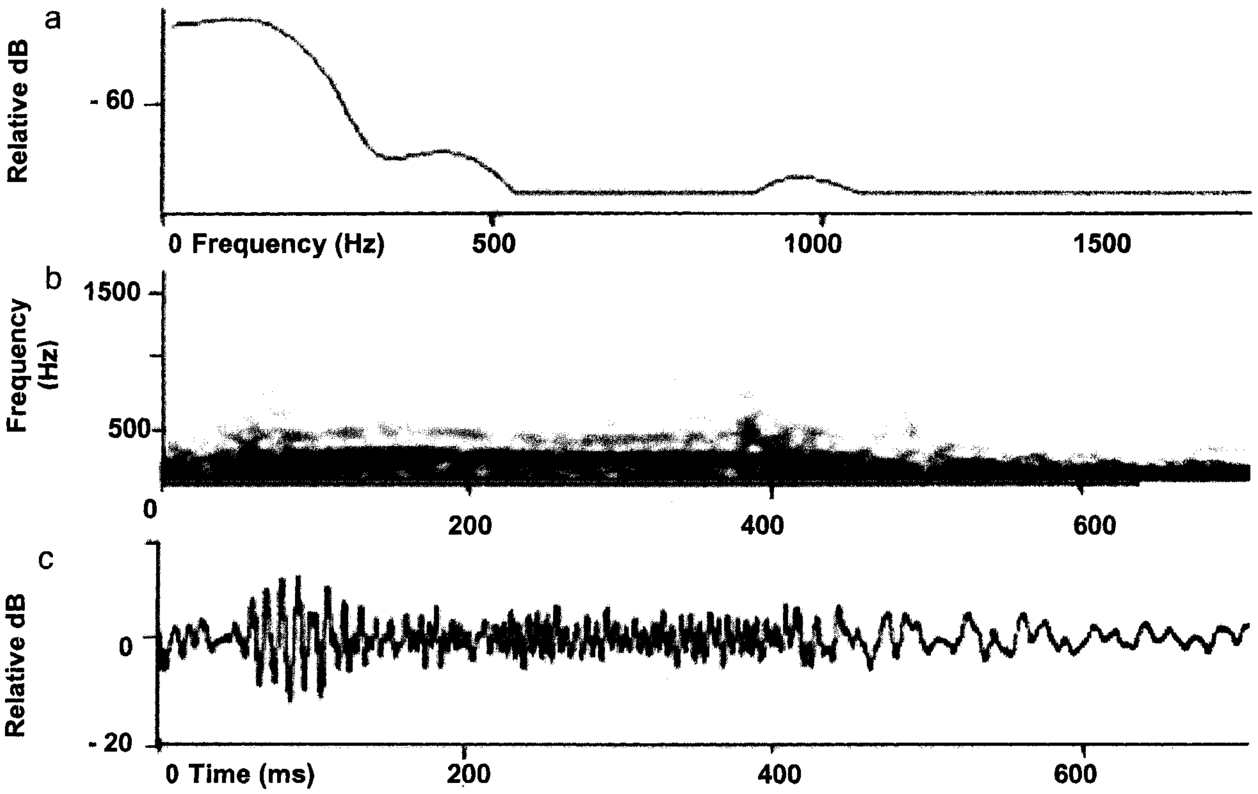


Figure 8: A power spectrum (a), spectrograph (b), and waveform (c), of a vocalization from a male *Etheostoma corona*. This species is shown as a relatively typical male vocalization, due to its relatively low frequency (under 100 Hz) and pulse-like structure. Relative dB is used as a non-unit measurement that shows the normalized power of the signal. These data are from Dr. Carol Johnston at the University of Auburn, Auburn Alabama.

that male vocalization rate was positively correlated with courtship rate (Amorim et al. 2003).

Sound Production Mechanisms

Contrary to other taxonomic groups such as mammals and birds which make vocalizations almost strictly by expelling air through a larynx or syrinx, fish have a wide variety of sound production mechanisms (Table 2) and many mechanisms are still unknown. One of the most common mechanisms of sound production in fish is the vibration of sonic muscles that are connected to the swim bladder once (which are referred to as extrinsic) or twice (which is referred to as intrinsic). These connections can be in various locations on the swim bladder. This mechanism results in a harmonic generally low frequency (below 1 kHz) signal. The overall dominant frequency is most often determined by the muscle contraction rate of the fish (Ladich 1997). Another mechanism is grinding together pharyngeal teeth or the strumming of pectoral (Family Ictalurade, Order Siluriformes) (Fine et al. 1997, Ladich 2001) or dorsal fin spines against other spines (Order Tetraodontiformes, Family Balistidae) (Salmon et al. 1969) These strumming and grinding mechanisms generally result in a more variable frequency (above or below 1 kHz).

Individual Recognition and Geographic Variation

With the exception of the extensive behavioral observations done on Italian gobies (*Padogobius bonelli* [formerly *Padogobius martensii*], *Padogobius nigricans* [formerly *Gobius nigricans*]) (Lugli 1997, Lugli et al. 1996, Lugli et al. 2001) and the neuroethological work done on the midshipman (*Porichthys notatus*) (e.g. Bass 1997, Brantley et al. 1993, Brantley and Bass 1994), the majority of work that has been

Table 2: Vocal fish and mechanisms of sound production if known

Vocal Species	Mechanism	References
Order Osteoglossiformes		
Family Mormyridae	Sonic swim bladder muscles	Yan & Curtsinger 2000
Order Batrachoidiformes		Gray and Winn 1961
Family Batrachoididae		
Opasanus	Intrinsic sonic muscles attached to Swim bladder	
Porichthys notatus	Intrinsic sonic muscles attached to Swim bladder	
Order: Perciformes		
Family: Pomacentridae	Unknown	Spanier 1979
Family: Gobidae	Unknown	Lugli et al. (1995) Rollo et al (2007)
Family: Sciaenidae	Sonic Muscles attached to the swim bladder	
Family: Cichlide	Possibly pharyngeal mechanism amplified by swim bladder	Lobel 1998 Lobel 2001
Family : Centrarchidae	Unknown	Gerald 1971
Lepomis (7 species)		
Family: Percidae	Unknown	
Ethostoma (2 species)		Johnston & Johnson 2000
Family: Serranidae	Unknown	
Family Osphronemidae	pectoral fins	Ladich et al. 1992
Family Cottidae	Muscles from skull to the pect girdle (no Swim Bladder)	
Family Haemulidae	pharygeal teeth	
Family Carangids	pharygeal teeth	Tavolga 1971
Order: Scorpaeniformes		
Family: Triglidae	Intrinsic muscles attached to the Swim bladder	Connaughton 2004
Prionotus		
Bellator		
Eutrigla		
Order Ophidiiformes		
Family: Carapidae	Slow sonic muscles attached to swim bladder	Parmentier et al. 2006
Order Cypriniformes		
Family Cyprinidae	Unknown	Johnston & Johnson 2000
Order Characiformes		
Family : Characidae	Unknown	
Order Gadiformes		
Family Gadidae	Paired sonic muscles attached to the Swim bladder	Hawkins and Amorim 2000
Order Siluriformes		
Family Callichthyidae	Unknown	Ladich 2001
Family Ictaluridae	pectoral spine locking	Fine et al 1997
Order Tetraodontiformes		
Family Balistidae	stridualtory appartus of dorsal fin	Salmon et al 1968
Order Cyprinodontiformes		
Family Cyprinodontiformes	Unknown	Johnson 2000

reported in fish vocalizations has been mainly descriptive in nature. However in recent years a number of studies have started to address other issues as well. Johnston and Johnson (2000b) investigated the sounds made by 3 species of darters. They characterized three distinct types of vocalizations, including a non-pulsed drum, a pulsed 'knock' and a 'purr' in each species. However they were able to determine that the duration and the fundamental frequency varied between species. Extensive studies investigating species-specific temporal variations have also been done in damselfish (Family: Pomacentridae) (Myberg and Ruggio 1985, Lobel and Mann 1995, Mann and Lobel 1997). Another source of variation of call structure may be fresh vs marine environments but there has currently been no research in this area.

Other non-descriptive studies that have recently begun to be addressed in recent years include the possibility of vocalizations being learned or innate (Johnston and Buchanan 2007) and the existence of geographic variation in call structure. Johnson (2001) investigated the possibility of geographic variation in the call structures of longear sunfish by recording from different sites and different sub-populations. Significant call variation was found at all the levels examined (individual, intra-site, inter-site, and within sub species) indicating a strong possibility of regional variation of call structure existing in this species.

The Family Gobiidae

The family Gobiidae is part of a larger sub-class of fishes known as Gobioidei. (Order Perciformes) which are also known as gobiioid fishes. In total this group accounts for over 2100 species in over 250 genera (Nelson 1994). The exact organization of this group is still debated, with the total proposed number of families in this group including two (Miller 1973), eight (Nelson 1994), and nine (Thacker 2000).

The family Gobiidae is primarily made up of hearing generalist fish including the subject of this thesis, the round goby (*Neogobius melanostomus*) (McCune and Carlson 2004) (Figure 9). The round goby is known to only hear sounds that are 600 Hz or less (Belangér and Higgs submitted). It has no swim bladder or other known accessory hearing structures. The round goby is a vocal species, however only the males are known to make a series of grunt-like, pulses during the reproductive season (Figure 10) (Rollo et al. 2007). Since males make these calls from their nest cavity, during the reproductive season, logic would imply that it would be important for females and possibly males to be able to localize these calls. However, the accepted theory on localization (Rogers and Cox 1988), suggests that a gas-filled structure such as a swim bladder is needed for localization. Contrary to this, previous research has indicated that these fish can behaviorally localize conspecific calls both in the lab and in the field (Rollo et al. 2007).

I investigated the mechanosensory system as a functional mechanism to allow round gobies to localize conspecific calls in a laboratory setting. I also investigated various temporal and functional characteristics of the call, to determine whether the vocalizations of males vary depending on hormone levels or individual morphological characteristics of the males.

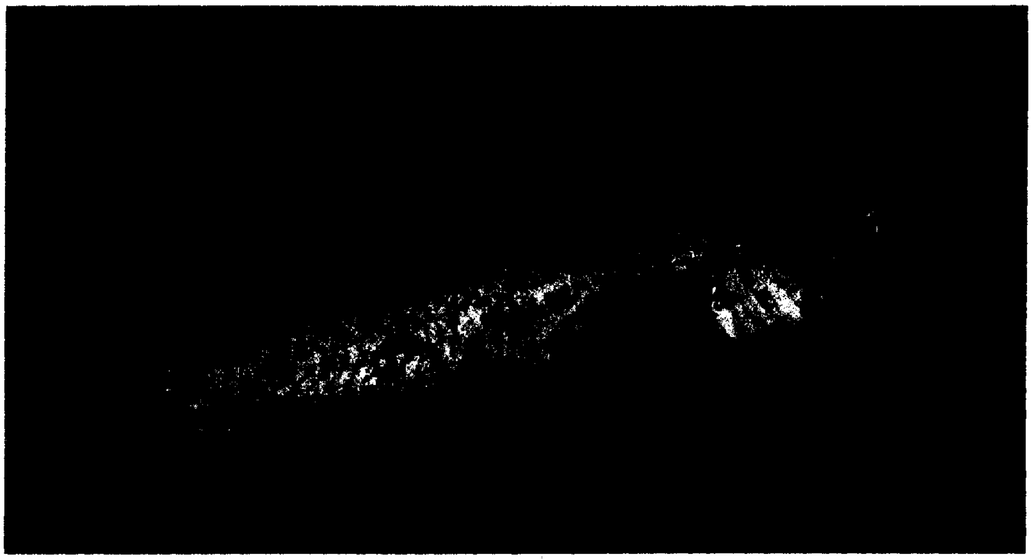


Figure 9: The subject of this thesis is the invasive species, the Round Goby (*Neogobius melanostomus*). Picture taken from: http://www.on.ec.gc.ca/green-lane/press/goby-invasion.html. Copyright to Environment Canada, 1999.

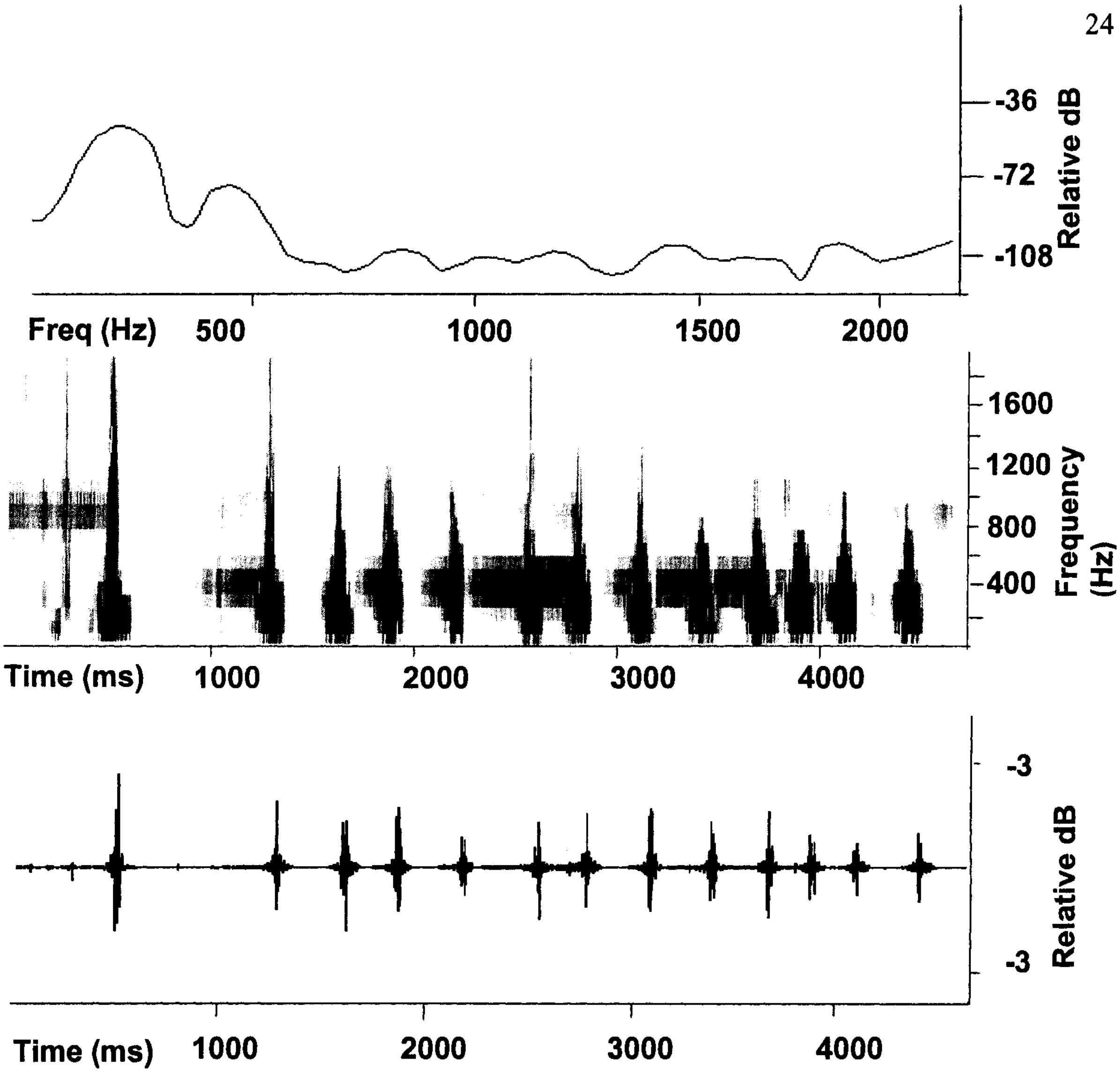


Figure 10: A power spectrum (a), spectragraph(b), and waveform (c), of a vocalization from a male *Neogobius melanostomus*. This call is composed of 'pulses' and is under 1000 Hz. Relative dB is used as a non-unit measurement that shows the normalized power of the signal.

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CHAPTER II

THE EFFECTS OF THE MECHANOSENSORY SYSTEM ON THE LOCALIZATION ABILITY OF THE ROUND GOBY (*NEOGOBIUS MELANOSTOMUS*)

Abstract

In murky and turbid waters, many sensory systems can be compromised, leaving acoustic communication essential for survival. However, many aspects of underwater acoustic communication remain poorly understood. In particular, research is needed to study localization abilities of hearing generalist fish, such as the round goby (*Neogobius melanostomus*). These fish have no accessory hearing structures, and theory would predict they would be unable to localize sound sources. It was recently found however that round gobies can localize conspecific calls. The current research investigates the role of canal and/or superficial neuromasts in localization. We played conspecific calls to control fish, fish that had ablated canal neuromasts and fish that had both superficial and canal neuromasts chemically ablated. Only fish with damage to both superficial and canal neuromasts took a significantly less direct path to the speaker than the other groups. These results suggest that superficial neuromasts are used in conjunction with ears to localize sounds. All fish behaviorally recovered their ability to localize sounds after 1 week. These results highlight the role of neuromasts in sound localization abilities of teleost fishes and the need for further study integrating multiple sensory systems.

Introduction

Throughout various taxa, vocalizations serve many purposes including territory defense (e.g. Lugli et al 1997) and mate recruitment (Reviewed by Hawkins 1993, Gerhardt 1994, Ladich 2004). While much is known about the importance of these vocal behaviors, often less is known about the sensory systems that allow animals to react to these signals. Many animals advertise a willingness to mate by vocalizing. However to be successful, a conspecific must have the ability to respond to that signal (i.e. locate the individual and respond accordingly).

In terrestrial animals the ability to localize a sound is dependent on both interaural differences (the difference in distance that the sound waves must travel to reach the tympanic membrane of each ear) and angle at which sound arrives at each ear (Brown and May 2005). However due to the small distance between the ears of fish, and the fact that sound travels up to 5 times faster in water than it does in air, the mechanisms that terrestrial animals use to localize sounds do not exist underwater (for details see Fay 2005). While some directional information can be gained from the complex organization and polarization of mechanosensitive hair cells in the end-organs of a fish's ear (Schuijf, and Siemelink 1974, Lu and Popper 2001) the accepted theory (Rogers and Cox 1988) suggests that accessory hearing structures in conjunction with a gas-filled sacs are needed for true sound localization. The gas 'sacs' (i.e. swimbladders or auditory bullae) transform acoustic pressure into displacement, leading to directional inputs to the ears (Schellart and Wubbels 1998, Rogers and Cox 1988). Despite the theoretical need for accessory hearing structures, there is evidence for directional hearing in generalist species (Myrberg and Spires, 1972), with at least one generalist species even being shown to localize sounds (Rollo et al. 2007).

A possible mechanism that has been hypothesized to allow fish to localize sounds is detection by the neuromasts of the mechanosensory system of fish (Rollo et al. 2007). Neuromasts, which contain mechanoreceptive hair cells, are characterized into two categories; canal or superficial. Canal neuromasts are found in shallow fluid-filled canals along the head or body of the fish while superficial neuromasts are located on the skin (Coombs et al 1992, Ahnelt and Scattolin, 2003). While similar in cellular structure, the two types of neuromasts exhibit differences in function, with canal neuromasts being more sensitive to acceleration (Montgomery et al. 1994, Coombs and Janssen 1990, Kroese and Schellart 1992) and superficial neuromasts primarily sensitive to changes in water velocity (Coombs and Montgomery 1994, Montgomery et al. 1994, Chagnaud et al. 2007). Also, canal neuromasts may be involved in orientation to a nearby stimulus (Coombs et al. 2001) and superficial neuromasts are responsible for rheotaxic responses (Montgomery et al. 1997, Baker and Montgomery 1999).

I investigated whether neuromasts are the functional link that allows the round goby (*Neogobius melanostomus*) to localize conspecific sounds. These fish have been shown to be capable of localizing sounds even without any obvious auditory specializations (Rollo et al. 2007). The neuromasts of each fish's mechanosensory system were selectively ablated using ototoxic antibiotics and their behavioral responses were tracked. The behavioral trials were also complemented by electrophysiological recordings (auditory brainstem response) of fish exposed to different treatments to determine the role of neuromasts on general auditory responses.

Methods

Sampling Methods

All fish used for these experiments were obtained from the Detroit River by seining or angling in relatively shallow water (c. 2 m). A 20 foot seine with ¼ inch mesh was used for the seining. The fish were transported using coolers and airstones to the lab which was about 5 minutes away (5- 35 fish were collected per trip). Once in the lab all of the housing provided was approved by the University of Windsor Animal Care Committee. All fish were tested within one week of being in the lab.

Antibiotics

To assess the ability of round gobies to utilize their mechanosensory system to localize sounds, antibiotics were used to selectively ablate the hair cells of this system. Fish were selected at random to be immersed in 0.02g L^{-1} gentamycin sulphate (24 hours), or 0.5g L^{-1} streptomycin sulphate (3 hours) (Appendix A). These particular antibiotics have been used in other experiments to ablate canal neuromasts (gentamycin) or both superficial and canal neuromasts (streptomycin) (Montgomery, et al. 1997, Song et al. 1995). Control fish were not treated with antibiotics but were transferred to tanks identical to those used to expose the other fish to antibiotics. The control fish were held in these tanks with identical conditions (i.e. light and temperature) for three hours prior to behavioral testing.

Experimental Procedure

Behavioral experiments were conducted in a 1020 L tank filled to a depth of 33 cm. Two speakers hung at one end of the tank 25.5 cm apart. The tank was bisected into an experimental arena that measured 140 X 92 cm, so that the entire arena could be seen by a video camera (Sony digital Handycam Recorder; model DCR-TRV27). A net was used to keep the fish within camera view and to cut down on sound reflectance, as sound waves were able to pass through the net but the fish were not. During the experiment one speaker was designated as the "live" speaker that would play a male round goby call and the other speaker was designated as the "silent" speaker. This "silent" speaker served as a control to ensure that all reactions of the fish included true localization (i.e. the fish were not just swimming in the general direction of the sound) and that the fish were not using the speaker as a shelter. The male round goby call used in the trials was obtained from John Janssen and Greg Andraso (see Rollo et al. 2007 for sound spectra details) and was approximately 4.5 s long but looped to play continuously. The sound level was adjusted to be approximately 150 dB re1μPa at the net and approximately 162 dB re1μPa at the speakers. This level was chosen because round gobies have been previously shown to respond to this level both physiologically (Belangér and Higgs submitted) and behaviorally (Rollo et al 2007). All trials in which the goby failed to react to the call were used to calculate the percent of fish that responded to the call but were not analyzed further. The trials in which the fish reacted were analyzed for reaction time, swimming speed, the time spent at the speaker and the distance ratio of the fish's swim path (described below). All reactions were tabulated by fishtracker software (Obtained from Dr. Lynda Corkum, and developed by Pauline Shen, University of Windsor).

Behavioural Measurements

Before the behavioral trials the fish were moved from a holding tank to the behavioral tank. The fish were transferred using a modified net that was made with a plastic bag instead of mesh. This allowed the fish to always have a "cushion" of water around them, assuring that there was little unintentional damage done to the neuromasts of the fish. Each trial began with a fish placed behind an arbitrary start line, 83 cm from the speaker. Once the fish was placed into the tank, the male call was played and the trial began. If the fish never moved during the trial then the fish was determined have no reaction to the sound. If the fish never passed the start line then the fish was determined to exhibit non-directional movement. Once a fish did pass the start line, their movements were characterized as being towards the silent speaker or the playing speaker. Once a fish swam away from the selected speaker the trial ended. All trials had a maximum duration of ten minutes.

The reaction time (s) of a fish was measured as the time from the beginning of the trial until the fish passed the start line. The velocity (cm/s) of the fish was determined from the time the fish passed the start line until it reached the speaker. The time(s) at the speaker measurement started when the fish first reached a speaker and ended when the fish left the speaker or when the ten minute trial ended. The distance ratio of the fish's swimming path was used as a measurement of how straight the fish's path to the speaker was. This ratio was established as:

$$\frac{\text{Hypothetical distance traveled (cm)}}{\text{Actual Distance traveled (cm)}} = \text{Distance ratio}$$

The hypothetical distance traveled was always 83 cm (distance from the starting line to the speaker) minus the distance the fish stopped from the speaker. The actual distance traveled was the sum of the distance traveled each second by the fish (as measured by fishtracker), from when they first crossed the start line until they stopped in front of their speaker of choice. If a fish swam in a straight path from the reaction line to the speaker then the path they took would have been 83cm, however most fish took a less direct path. A ratio of one indicates straight line swimming while a ratio less than one indicates a less direct path. This measure was critical in determining if the fish were using a straight localization technique or if they were taking a less direct path and sampling sound fields to determine the origin of the playing sound. This estimate differs from previous measurements of localization used by our lab (Rollo et al. 2007) due to changes in lab software, but we feel that it represents a comparable measurement of localization ability.

Behavioural Recovery

Originally all fish were retested after 1, 2 and 3 weeks to determine if any behavioral differences seen between the treatment groups would change with the recovery of the neuromasts. After it was found that all fish were able to behaviorally recover after 1 week (about half way through the behavioral trials) the remaining fish were only retested after a 1 week period of recovery. A 1 week recovery period is consistant with other studies that concluded morphologically hair cells appear to return to normal appearance after 1 week (Song et al. 1995)

Auditory Brainstem Response

To test the physiological effects of neuromast ablation, fish from each treatment group were tested using an auditory brainstem response (ABR) setup. The fish that were treated with antibiotics were then retested one week later to investigate the physiological recovery of the fish. The setup used for the ABR has been previously described in Wright et al. (2005) and, Bélanger & Higgs (submitted) and therefore will only be described briefly here. Recording and reference electrodes (Rochester Electromedical, Tampa, FL USA) were placed under the fish's skin, with the reference electrode near the nose of the fish and the recording electrode along the medial axis of the body in line with the beginning of the pre-opercle. This spot was shown to correspond with the auditory brainstem of the fish in previous dissections. The tone bursts played to the fish ranged from 100 to 600 Hz (intervals of 100Hz) and 100 to 150 dB re1 µPa (intervals of 5 dB re1 µPa). Tone bursts were played through a completely submerged underwater speaker

(UW-30, Lubell Labs Inc. Columbus, OH USA) to fish which were 0.76 m away from the speaker. The sound was gated using a Hanning window, filtered between 40 Hz and 10,000 Hz, and notched at 60 Hz to eliminate electrical background noise. The response of the fish was measured to determine the auditory threshold (dB re1 µPa) for each fish at the various frequencies. For each frequency the sound level was increased by 5 dB re1 µPa until a stereotypical ABR was detected. The testing continued for at least 10 dB re1 µPa above threshold. All detection was done by a visual assessment as this has been shown not to differ from a statistical approach in other studies (Mann et al. 2001).

Microscopy

Since the antibiotic treatments have been used successfully on other species of fish but not on round gobies, dissections were done on the ears of fish to ensure the quality of the hair cells in the ears were left intact. The sensory epithelia of the saccules of the ears were extracted from the head of the fish after being fixed for 24 hours in 4% paraformaldehyde. The specimens were stained with phalloidin, (Oregon Green, Molecular Probes, Oregon USA) (Appendix B) and examined under a fluorescent microscope (Zeiss Axioskop 2 fluorescent microscope) under a magnification of 10x and 20x. At no time did we see any evidence of damage, with epithelia of fish from all treatments being identical and complete.

Statistical Analysis

To determine if there was a statistical difference between the amount of fish responding to the playing or silent speakers, a χ^2 analysis was run, for each treatment. A one-way ANOVA was run to test for differences in the swimming velocity, the distance from speaker, time spent at the speaker, and reaction time between the three treatments (control, gentamycin treatment or streptomycin treatment) for all fish which responded. The distance ratio data were normalized by taking the arcsine of the square root of the distance ratio proportion (Zar 1984). The data were then tested for normality using a one sample Kolmogorov-Smirov test, which found these data to be normal. A one-way ANOVA was then run on the transformed distance ratio data, as well. If the ANOVAs revealed any significant differences between the treatment groups then a *post hoc* bonferroni adjusted pairwise comparison was run to determine where the differences were found. A two-way ANOVA, was used for looking at the effect of frequency and treatment on the threshold of the fish was run on the data obtained from the ABR test. Once again a *post hoc* bonferroni adjusted pairwise comparison was also used to test for differences in this ANOVA. All ANOVAs were run using Systat 10 (SPSS Inc., Chicago IL USA).

Results

Overall 67 (15 female, 52 male) control, 50 (9 female, 41 male) streptomycin and 49 (2 female, 47 male) gentamycin fish were tested. Of those only 32 (48%; 7 females, 25 males) controls, 35 (70%; 7 females, 28 males) streptomycin fish and 40 (82%; 3 females, 37 males) gentamycin fish showed any movement at all during the trial.

Of the responding fish 56% (18 fish; 5 female, 13 male) of the controls, 60% (24 fish; 3 female, 21 male) of the gentamycin and 57% (20 fish; 5 female, 15 males) of the streptomycin fish exhibited a preference for one of the speakers, by crossing the arbitrary 83 cm line (towards the playing or silent speakers). It should be mentioned that even though a higher percentage of antibiotic treated fish showed movement during the trial, the actual percentage of fish that moved towards the speaker was between 50%-60% for all treatment groups. The remaining fish exhibited non-directed movement. For the control group, 11 (3 females, 8 males) fish preferred the playing speaker and 7 (2 females, 5 males) preferred the silent, of the gentamycin treated fish 11 (1 female, 10 males) preferred the playing speaker and 13 (2 females, 9 males) preferred the silent and in the strep group there was higher preference for the silent speaker (13 fish; 4 females, 9 males) than the playing speaker (7 fish; 1 female, 6 males). A χ^2 test looking at all of the treatments was not significant. Thus the response of fish to either speaker was random. However there was a definite trend present in which the preference for the playing speaker decreased with neuromast ablation (Figure 11).

One-way ANOVAs determined that there was no statistical difference between the reaction time (control n =11, gentamycin n =11, streptomycin n =7; df =2; F=0.663, p=0.655) the amount of time spent at the speaker (control n =11, gentamycin n =11, streptomycin n =7; df =2; F=0.399, p=0.846) the velocity(control n =11, gentamycin n =11, streptomycin n =7; df =2; F=0.514, p=0.764) or the distance from the speaker (control n =11, gentamycin n =11, streptomycin n =7; df =2; F=0.595, p=0.704) between any of the treatments (Figure 12 A,B,C, and D respectively). There is a possible trend of control fish going closer to the speaker (Figure 12 D) and taking longer to react (Figure 12 A) however these differences were not significant. It also appears the gentamycin-treated fish spent less time at the speaker (Figure 12 B) and possibly traveled faster (Figure 12 C), however once again these differences were not significant.

There was a significant difference in the distance ratio for the treatments (Figure 13). Fish that were treated with streptomycin (i.e. lacking both superficial and canal

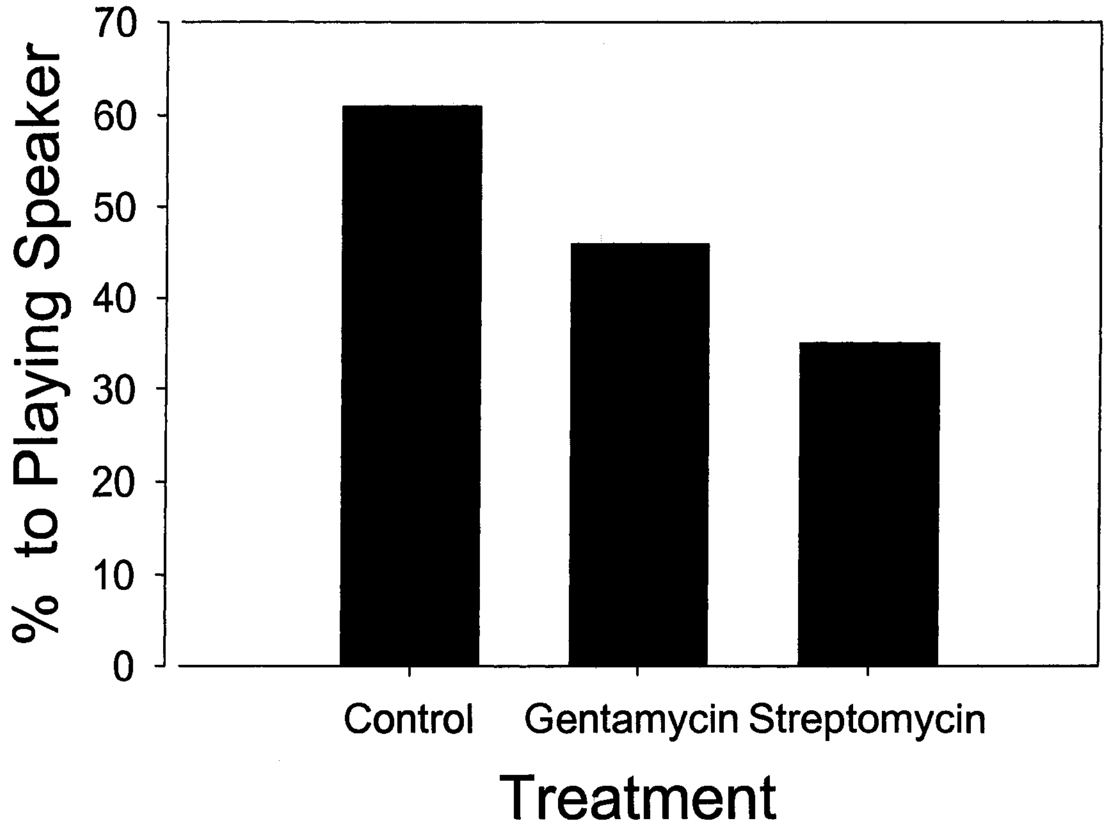

Figure 11: The percent of fish responding to the playing speaker for each of the three treatments. The highest percent of fish responded to the playing speaker when no neuromasts were damaged(11/18 fish), a lower percent of fish responded with the gentamycin treatment (damaged canal neuromasts)(11/24 fish) and the lowest percent of fish responded with the streptomycin treatment (damaged canal and superficial neuromasts) (7/20).

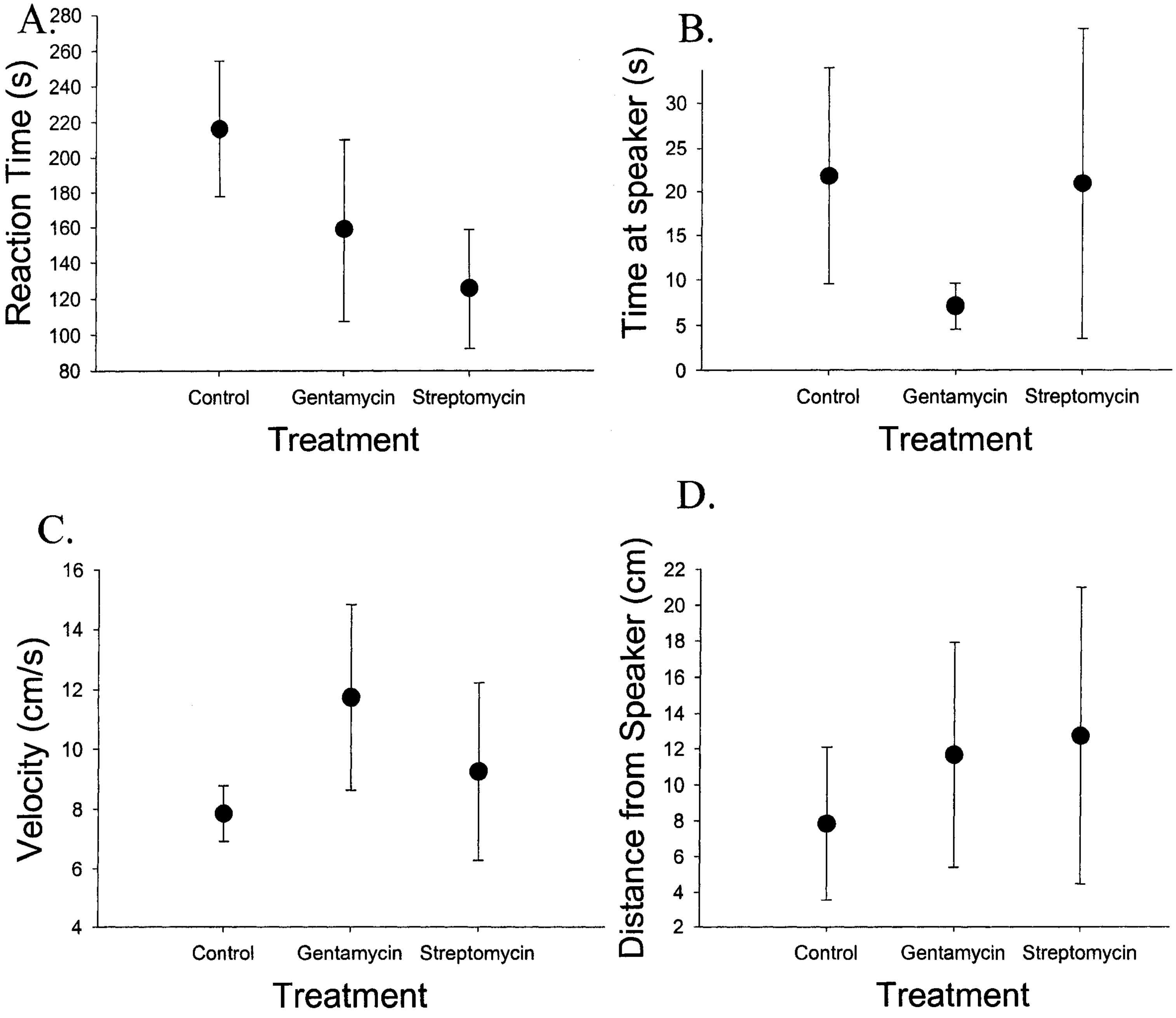

Figure 12: There was no statistically significant difference in the (a)reaction time; (b)time to speaker; (c)velocity; or (d) the distance from the speaker for the three treatments. This shows that the antibiotics did not affect the majority of the behaviours that the fish exhibited therefore the fish most likely did not suffer any ill effects of the antibiotics.

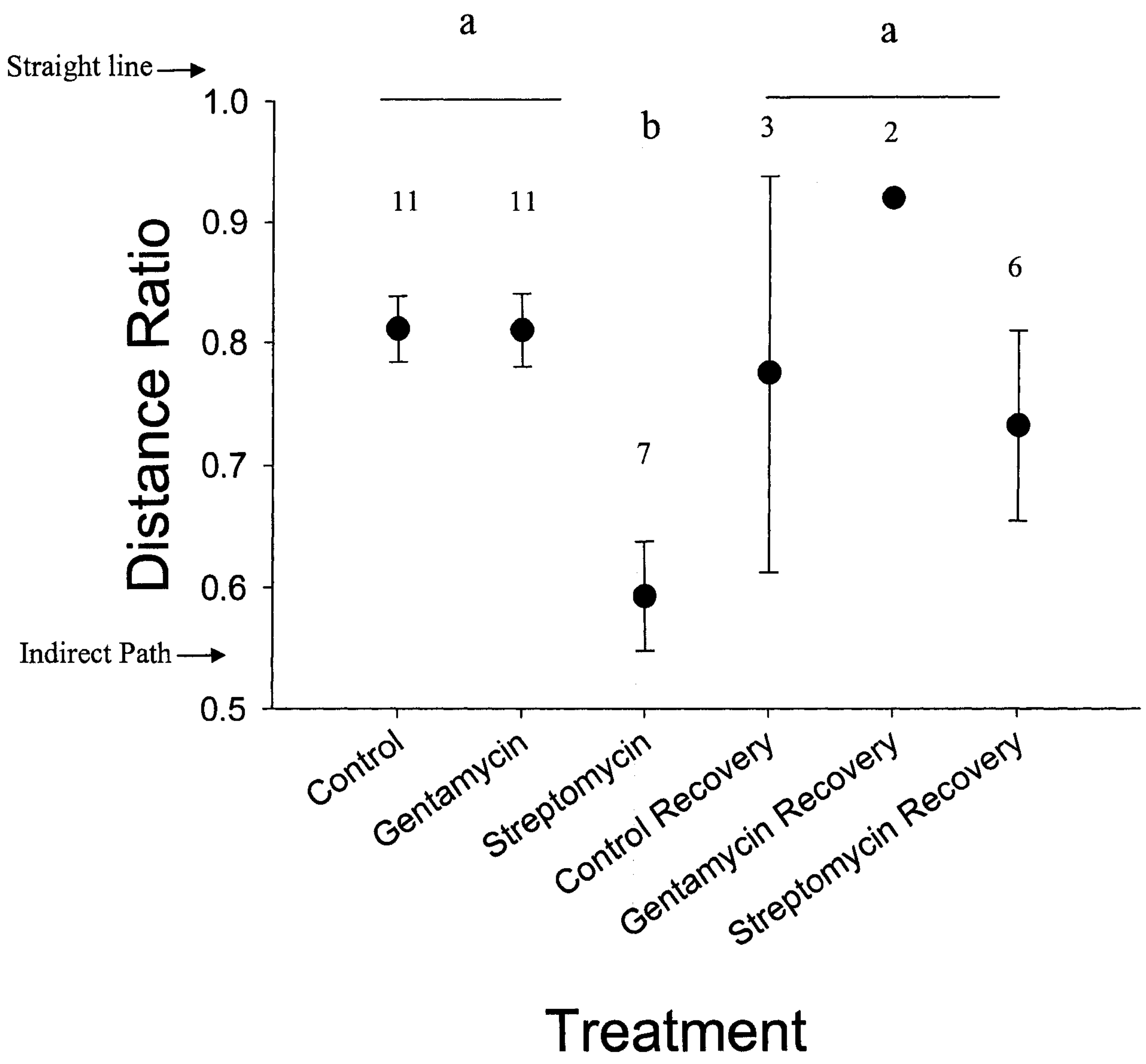

Figure 13: The distance ratio of the streptomycin treated fish (ablated superficial and canal neuromasts) was significantly lower than that of the other groups (thus they took a longer path). However after being given a week to recover the distance ratio of the streptomycin treated group returned to the same level as that of the control fish and the gentamycin treated (those with damage only to their canal neuromasts) fish. Only the non-transformed data are plotted on the graph.

neuromasts), took a significantly longer path (i.e. a smaller distance ratio) than the control fish and the gentamycin fish (control n =11, gentamycin n =11, streptomycin n =7; df =2; F = 4.416; p=0.004) (Figure 13). Gentamycin fish (i.e. damage to their canal neuromasts only) took the same relatively straight path as the control fish. After a 1-week recovery period, the distance ratio of the streptomycin fish returned to the level equal to that of the controls (Figure 13). This indicates that with recovery of both superficial and canal neuromasts, the fish's ability to localize sounds is no longer impaired.

When the fish chose the silent speaker, there was no significant difference between the treatments including recovery, for the distance ratio (N=44, df = 5, F=1.196, p=0.330), time to speaker (N=44, df = 5, F=0.813, p=0.548), distance from speaker (N=44, df = 5, F=0.805, p=0.554), the time at the speaker (N=44, df = 5, F=0.218, p=0.956), and velocity (N=44, df = 5, F=1.295, p=0.256) between all treatments. There was also no significant difference between the distance ratio of the fish that traveled to the silent speaker compared to that of the control and gentamycin-treated fish that traveled to the playing speaker (p=0.80) indicating that they all took the same relatively straight path to the speaker.

Auditory Brainstem Response

The auditory brainstem response showed that the fish treated with streptomycin (n=3) and gentamycin (n=4) had a significantly higher overall threshold (lower sensitivity) then the control fish (n=5) (df =2, F=.15.643, p< 0.001). Within the control group and the streptomycin group there was no overall difference in thresholds between frequencies (control: n=5, df = 5, F=1.333, p=0.284; streptomycin: n=3, df =5, F=0.911, p=0.506).

There was a significant effect of frequency on thresholds within the gentamycin group (n=4, df = 5, F =3.288, p=0.028). However a *post hoc* bonferroni adjusted pairwise comparison revealed no individual differences among frequencies (Figure 14).

After a one week recovery period there was no significant difference between thresholds of the control group and the gentamycin group. This indicates that given enough time for the canal neuromasts to recover the threshold can return to the level it was before treatment. However after a week of recovery the threshold of the streptomycin group was still significantly above that of the controls (Figure 15).

Discussion

This study examined the role of superficial and canal neuromasts in the ability of the round goby to localize a sound source. It has been previously shown that the lateral line system is involved in responses to sound sources at close distances (i.e. within one body length of the fish) (Coombs and Conley 1997a, Coombs and Conley 1997b, Coombs et al 2001) and it has even been stated that the lateral line cannot be used to detect stimuli at greater distances than 1-2 body lengths (Coombs and Montgomery 1998). However in a natural setting there is an obvious need to detect stimuli such as prey, predators or possible mates from much greater distances, as in the current experiment. This study found that both the superficial and canal neuromasts contributed to the fish's ability to distinguish and orientate themselves between the silent and playing speakers (Figure 1). I showed that the damage to the canal neuromasts impaired this ability, further ablation to both systems caused further impairment.

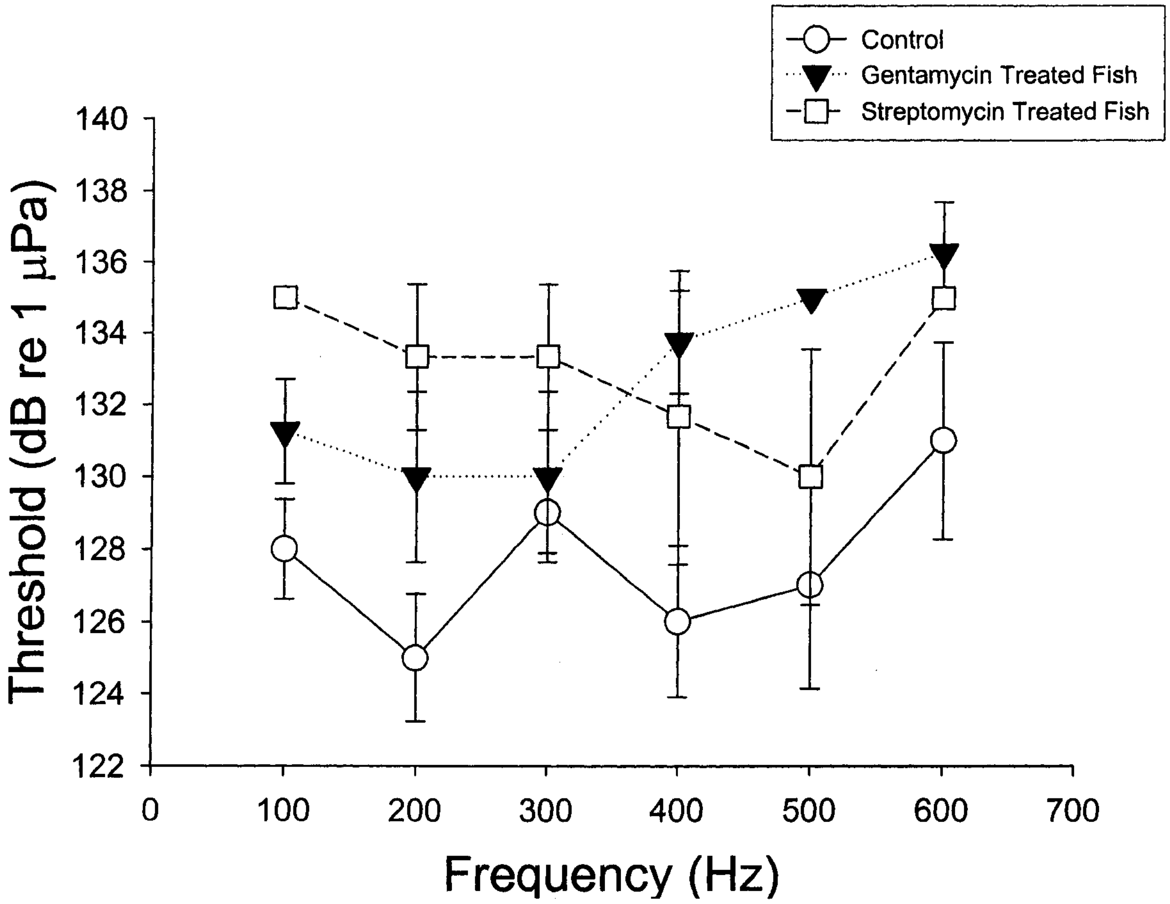

Figure 14: All fish treated with streptomycin (ablated superficial and canal neuromasts) and gentamycin (fish with damage to canal neuromasts) had a significantly higher threshold than the control fish (no damage to any neuromasts). The axes are minimum threshold as measured in decibels, a measurement of pressure, relative to 1 μ Pascal, and the frequency of the pure tones played, measured in Hertz

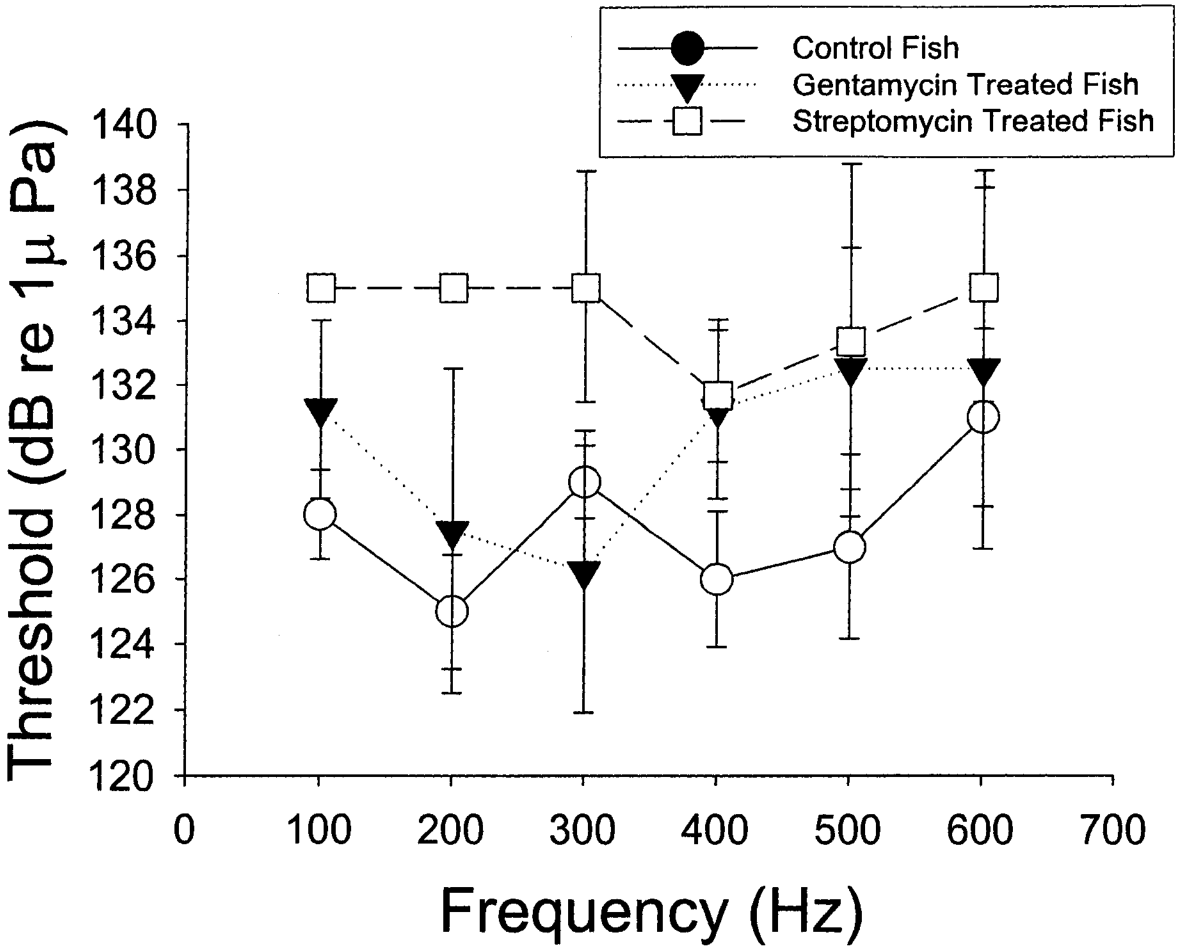

Figure 15: After a week to recover from the antibiotic treatment the threshold of the gentamycin treated fish was not different from that of the control fish. However the streptomycin treated fish still had a significantly higher threshold. The axes are minimum threshold as measured in decibels, a measurement of pressure, relative to 1 μ Pascal, and the frequency of the pure tones played, measured in Hertz

By examining the fish's response from a further distance from the speaker, we were able to examine not only if the fish responds 'correctly' (i.e. to the playing speaker) but also, how the fish responds to the sound source. The current study concludes that superficial neuromasts are involved in localization in the round goby. Fish with only damage to canal neuromasts (gentamycin fish) were less likely to choose the correct speaker, but once that speaker was determined they were able to take a similar path to the sound source as the control fish. Once a fish started to swim towards the speaker, the fish depended more on its superficial neuromasts to localize sounds. In the absence of these superficial neuromasts the fish took a more indirect path (i.e. a significantly lower distance ratio) which is indicative of not being able to localize the sound source. Instead, these fish seem to adapt the strategy of sampling sound fields to find the object.

Superficial neuromasts have been shown to be more sensitive to low current velocities than the canal neuromasts (Coombs and Montgomery 1994, Montgomery et al 1994). Changes in particle motion as the fish moves closer to the sound source may have been strong enough to stimulate the superficial but not the canal neuromasts. Moreover, it has been shown that each individual neuromasts is innervated by separate neurons that are not connected to any other neuromast (Münz 1985). This could allow the neuromasts to act as a receptor array detecting different velocities at different points of the head and body. The head of the fish, being closer to the sound, would receive the sound source at a slightly stronger level than the rest of the body which is farther from the sound source. Unilateral denervation of both canal and superficial neuromasts does not inhibit mottled sculpin (*Cottus bairdii*) from localizing a sound source (Conley and Coombs 1998).

However, partial denervation of one side (i.e. denervation of only the neuromasts on the head but not the body) caused a decrease in the sculpin's ability to orientate to the stimuli on that side, supporting our results discussed above. Further support for the hypothesized role of superficial neuromasts in sound localization is the ability to recover localization abilities in 7 days. This amount of time is similar to the recovery period needed as reported in other morphological studies (Song et al. 1995).

A potential critique of behavioral studies following neuromast ablation is that fish are sick due to treatment effects (Janssen 2000). The lack of a significant difference in the behavioral measurements between groups in this study shows that the fish were not exhibiting 'disordered behavior' due to the treatments, therefore the differences in responses that were shown to be significant were most likely due to the absence of the respective neuromasts and not a side effect of the ototoxic treatment.

The behavioral responses of the fish in this study were comparable to previous studies done looking at the localization ability of this hearing generalist fish (Rollo et al 2007). The previous study used the standard deviation of the mean path angle to show localization ability, however in the current study the distance ratio measurement was used. Both methods of measurements showed that under normal conditions (i.e. without antibiotic treatment, control fish in the current study, and all fish in Rollo et al. 2007) the fish took a direct path to the speaker. None of the fish in the previous study were treated with antibiotics and at no time did these fish show an inability to distinguish between the two speakers as those treated with antibiotics or the 'zig-zag' pattern, exhibited by the streptomycin treated fish.

Another critique of this study might be that all behavioral experiments were done in a lab setting which among other things, lacked any flow or ambient noise. This means that the environment in which we tested the fish's ability to localize sounds was one that they would never encounter in the wild. However, their reactions to the sounds in this environment were adequate for this study. Round gobies have been shown to respond to conspecific male calls with a more direct path and with a greater velocity than in comparison to heterospecific calls in this same lab setting (Rollo and Higgs submitted). Their have been other attempts to track the response round gobies to the same male call in the Detroit River (Windsor, Ontario, Canada) however, due to poor visibility these attempts were not successful.

Overall the hearing threshold of the controls was lower than that of the treatment groups, as determined by the ABR. This could be an indication of a loss of hearing abilities, due to the treatments effect on the ears of the fish, or it is possible that the lateral line response contributes to the overall response being picked up by the ABR. Since there was no damage to the hair cells in the saccule of the fish, as shown by the confocal microscopy, it can be assumed that the hearing abilities of the fish were not compromised. Both superficial and canal neuromasts, as well as the auditory nerve (VIII), all innervate the brain at the brainstem (McCormick 1999, Higgs et al 2006), near the recording site for ABR. The use of ABR probably detects both ear and neuromasts responses. This rise in threshold is indicative of the antibiotics causing the mechanosensory system to be physiologically ablated. After a recovery period of a week, the streptomycin fish did not recover back to the same threshold level as the controls, however the gentamycin treated fish did appear to recover. One reason for this may be because all fish received some damage to their superficial neuromasts while being restrained for the ABR procedure. Although, we attempted to handle the fish as little as possible some damage was unavoidable and should be assumed to be equal for all treatments. However, the control and gentamycin recovery fish could have had more intact superficial neuromasts to start with considering that their treatment did not affect these neuromasts. However the superficial neuromasts of the streptomycin fish did receive antibiotic damage. It is possible that damaging a few superficial neuromasts on the streptomycin fish could have partially or fully negated any recovery that had taken place, thus resulting in a higher threshold.

Due to equipment constraints, the ABR set up we used in the current experiment measured only the pressure component, and not the displacement of sound. This could be seen as problematic for a hearing generalist fish, which has no known pressure sensitive structures such as swim bladders. However, this set up is adequate enough to fulfill the purpose of this study, which is to show that there is an effect of antibiotic treatments on the overall 'noise detection' of the round goby.

This study shows that round gobies cannot behaviorally localize a sound without superficial neuromasts. However, we should recognize the overall importance of multisensory integration in the total localization response. All teleost fish are able to detect particle motion through the movement of sensory epithelia, relative to otoliths in their ears, allowing the fish to determine the relative but not direct origin of the sound source. However ears are sensitive to the displacement component of sound that is not adequate to allow for localization alone (Schellart and Wubbels 1998). The canal neuromasts are also important to the overall localization response, as shown by this and other studies (Coombs and Conley 1997a, Coombs and Conley 1997b, Coombs et al 2001). However, the current research points out that once the fish had oriented to the sound, even a hearing generalist fish without intact canal neuromasts could swim directly toward a sound source. These results demonstrate insight but also a further need to understand the role of multisensory integration not only with regards to the mechanosensory system but in all aspects of the communication abilities of teleost fishes.

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CONCLUSIONS AND RECOMMENDATIONS

Thesis Relevance

This thesis has focused on the ability of the round goby to both respond to and make vocalizations however, it has relevance in other areas of study. The localization of sound has often been attributed to the presence of accessory hearing structures (von Frisch 1938). However, fish such as the round goby which have no specializations have been overlooked. I found that the neuromasts of the mechanosensory system, and especially the superficial neuromasts are responsible for allowing these hearing generalist fish to localize sounds. The localization of sounds such as the reproductive vocalizations used in this study, is essentially important for successful mate choice and reproduction. Therefore, the findings of this thesis add greatly to our overall knowledge of fish behavioural ecology.

However, as always, along with this increase in knowledge there also are questions that arise from these findings. One such area of future investigation may be the importance of the mechanosensory system to hearing specialist fish. There have been studies that show that fish with swim bladders (hearing specialists) use these structures to localize sounds (Schuijf and Siemelink 1974, Schellart and Wubbels 1998). However, these fish also possess a mechanosensory system, which as this thesis proposes can also be used to localize sounds. A possible future study could look at a hearing specialists ability to localize sounds, with both an intact swim bladder and mechanosensory system, and then with either or both systems ablated. If these fish are unable to localize sounds with a deflated swim bladder and an intact mechanosensory system, (as suggested by previous research; Schellart and Wubbels 1998) than this may suggest differences in neuromasts and/or lateral line structures that are currently not known.

Currently, much research is being performed in the area of fish vocalizations. In particular, studies on passive acoustic localization are being used to determine where fish live, feed and spawn. However, there are still many areas that should be investigated in the area of sound reception by fish. It is unknown if many fish are vocal or not, and the actual mechanism that allows many fish that are vocal to make sounds is still unknown for many species (Ladich 2004). In order for any future studies to be successful, we must first do investigative studies to determine the characteristics of various calls made by the different species of fish such as was done in this thesis.

There are still farther reaching indirect implications of this study. One area that might be of particular relevance to the medical community is the exact mechanism that allows for the regeneration of the hair cells of the mechanosensory system. We examined the behavioural recovery period of these neuromasts; however, we did not investigate the actual mechanism that allows for their regeneration. Since many vertebrates have these sensory structures, finding the mechanism that allows regeneration in birds and fish would lead to many medical advances in areas such as hearing loss and deafness.

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APPENDICES

APPENDIX A

A preliminary investigative study of vocalization abilities in the round goby (*Neogobius melanostomus*)

Fishes, like other vertebrates, have a variety of different sensory systems used to gather information from the world around them. Acoustic communication is one such modality (For a review see Ladich 2004; Amorim 2006). It is known that male round gobies (*Neogobius melanostomus)* are vocal during the reproductive season (Protasov et al. 1965, Rollo et al. 2007). In the current investigative study, male round gobies were injected with luteinizing hormone-releasing hormone (LHRH) to examine the effect of hormone levels (i.e. possibly reproductive state) on the call structure of this species. We used the assumption that the LHRH injections would increase the level of hormones present in each fish. We also assumed that the amount of hormones in the fish would decrease as time post injection increased. However, since this study was solely investigative, we did not do any direct tissue sampling, nor did we attempt to assess the reproductive status of the males. Without this information this study is still valuable because variation of sound structure has not been previously investigated and is not entirely understood in this species. Therefore, in addition to investigating the effects of hormones on the structure of male vocalization, we also investigated if inter-individual differences existed in the call structure.

Methods

Round gobies were collected during the summer and fall of 2006 by angling and seining in the Canadian waters of the upper Detroit River. They were stored in groups of up to 30 individuals for up to three months in the animal facilities at the University of Windsor. Fish were maintained in aerated, 16 - 18°C dechlorinated tap water, kept at a 16:8 light/dark cycle and fed Nutrafin® fish flakes (Tetramin, Inc.). Once an experimental fish was injected, it was kept isolated from the rest of the fish. The fish used in the trials varied in size from 14.21 g to 39.68 g and 105 mm to 132 mm in total length. All housing and experimental procedures reported in this study were approved by the University of Windsor Animal Care Committee, in compliance with guidelines established by the Canadian Council of Animal Care.

LHRH Injection Protocol

Every three weeks a single male round goby was injected with luteinizing hormone-releasing hormone (LHRH) (Sigma Aldrich Company, St Louis, MO, USA, product #: L4513). Before injecting the fish with LHRH, both the weight (g) and the total length (TL) (mm) were recorded. Each male was injected with 0.1mg LHRH per 50g of fish on one side of the dorsal muscle (Appendix D). Injections were repeated every 4 hours, for a total of 3 injections per day (Protocol for LHRH injections obtained from Dr. Lynda Corkum, University of Windsor). Between the injections the fish were kept in a separate tank between 20-24°C.

Recording Set-up

At the beginning of each recording session, 30 seconds of 'silence' were recorded in the tank to determine the level of the background noise in that tank. This was later used to filter out this background noise. To illicit vocalizations from the experimental fish, we used a playback technique in which we played a looped recording of a male round goby call at 150 dB re1μPa for one minute. This was followed by 90 seconds of silence and then the call was played for another minute. Recording of the new vocalizations then continued for an additional 25 minutes. The entire recording procedure was then repeated, so that two, thirty minute tracks were recorded each week. All recordings were done on Thursday mornings from 9-11am. A DAT-Recorder (Marantz Inc. Model # PMD670) connected to a Reson TC4032 hydrophone (Reson Inc, USA) was used to record calls. The male round goby call was played in the tank using a portable CD player (Duraband, Lenoxx Electronics Corp, Carteret NJ, USA) connected to an Alesis RA300 amplifier (Alesis Studio Electronics, Santa Monica, California, USA) through a fully submerged UW-30 underwater speaker (Lubell Labs Inc, Columbus OH, USA). Before each recording the temperature of the tank was noted. Also the physical appearance (i.e. coloration) and behavior of the fish was recorded during each trial.

Track and Individual Call Analysis

It is necessary to first explain the terms and procedures used in analysis of these calls since there was has been little work done describing the characteristics of this call. This previous work (Rollo et al. 2007) was strictly descriptive and did not attempt to correlate call structure with any individual fish characteristics. The work that has been done in other species is highly variable (For a description of the various terms used in analysis see Kihslinger and Klimley 2002). For the purpose of this paper, comparisons with previously recorded calls were made to help understand what exactly was being looked for. Since the original call used for comparison was approximately 129 Hz, all sounds consisting of a series of pulses between 80 and 300Hz were saved for later call analysis. The first step in analyzing the calls was to distinguish a 'pulse' from what was background ambient noise. First it was determined how many pulses could be heard by the researcher and each of these beats were then matched to a peak on the waveform and each peak was determined to be equal to one pulse. The amount of time between the highest point of one pulse to the highest point on the next pulse (as determined on the waveform) constituted the 'inter-pulse' or 'inter-call' intervals. The term 'call' was used to define multiple pulses grouped together and a duration greater than 1000 ms between pulses constituted a new call. Only calls with more than one pulse were analyzed. The term 'track' was used to describe one thirty minute recording session. All thirty minute tracks were analyzed using *Adobe Audition* (Adobe Systems Inc., San Jose California, USA). In total there were six different properties of the call that were used to analyze the sounds: 1) the number of pulses present in each call, 2) number of pulses per thirty minute track, 3) the number of calls per thirty minute track 4) all inter-pulse durations and 5) all inter-call durations 6) the fundamental frequency of the call.

Statistical Analysis

The mean and standard error for all of the above call characteristics were taken, comparing the tracks 1 day, 1 week and 2 weeks post injection to measure the effect of hormone level on the call structure. The calls of each individual fish were also compared to determine if there was any individual variation between the males. Six different 2-way ANOVAs were performed in SYSTAT 10 (SPSS Inc. Chicago IL, USA) with male identity and week number as factors and each of the characteristics of the call being a dependent variable in a separate ANOVA. If statistical variation did exist then a *post hoc* bonferroni adjusted pair wise comparison test was also performed. Fish that did not produce calls every week (weeks 1, 2, and 3), were excluded from the statistical analysis.

Results

The analysis of variance for inter-pulse duration showed that there were significant effects of individual variation (n = 4, df = 3, F = 3.798, p = 0.010). A *post-hoc* Bonferroni test revealed a significant individual variance between fishes 1 and 3 and between fishes 1 and 6 (Figure 16A). There were no effects of hormones between weeks one through three (n = 3, df = 2, F = 0.878, p = 0.416) (Figure 17A). There was, however, a significant interaction factor between male number and week number (n = 7, df = 6, F = 20826, p = 0.010). The number of pulses per call ANOVA also showed significant effects of individual variation (n = 4, df = 3, F = 3.538, p = 0.015) (Figure 16B). A *post-hoc* Bonferroni test showed significant individual effects between fishes 1 and 2. There were no significant difference between weeks (n = 3, df = 2, F = 2.178, p = 0.114) (Figure 16B) but there was a significant interaction factor (n= 7, df= 6, F= 10.613, p < 0.001).

Looking at frequency, statistical analyses illustrate no significant effects between individuals (n = 4, df = 3, F = 2.583, p = 0.052) (Figure 16C), no hormone effects (n = 3, df = 2, F = 1.283, p = 0.278) (Figure 17C), and no interaction effects (n = 7, df = 6, F = 1.810, p = 0.095).

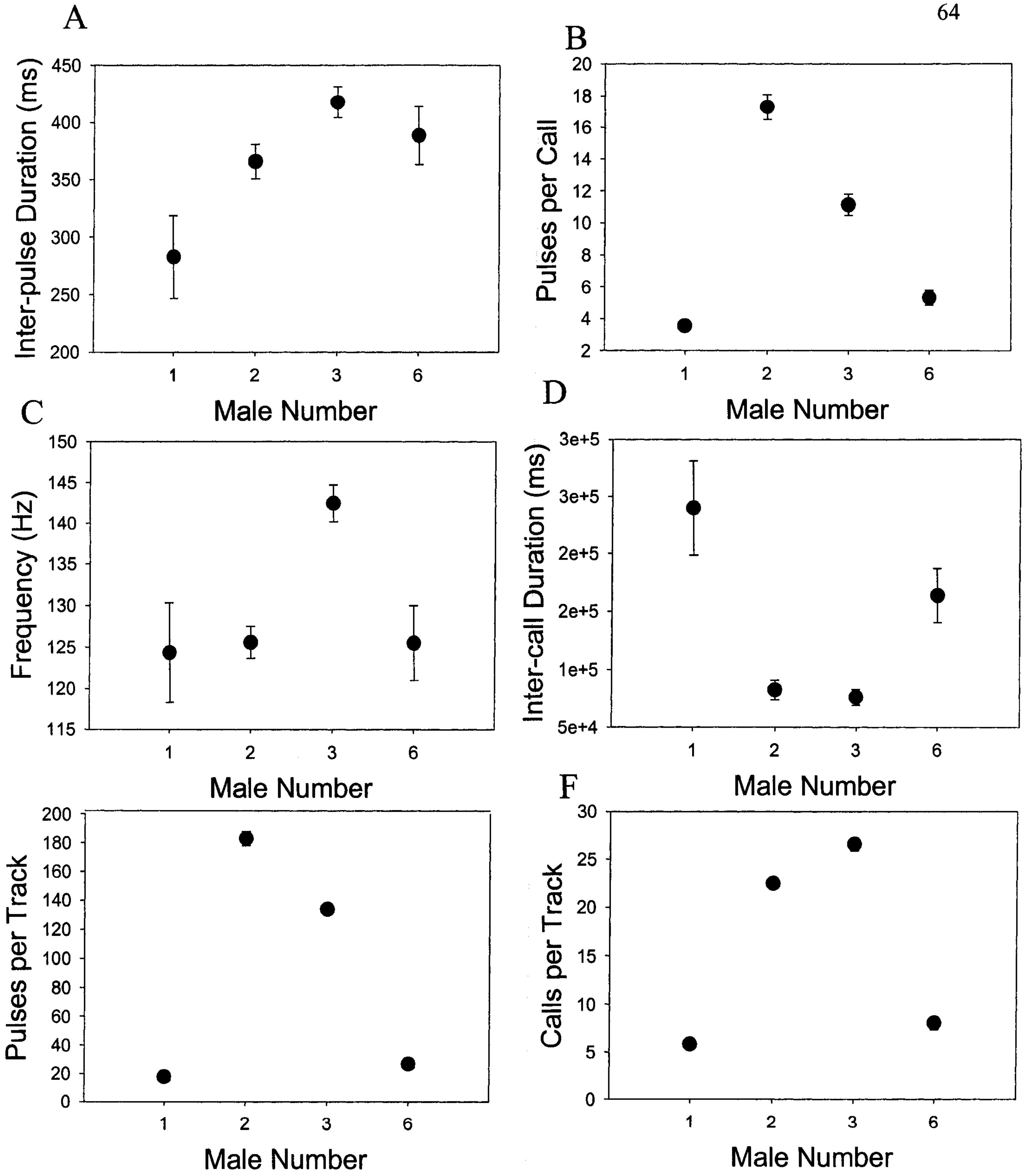

Figure 16 The individual differences in call structure between male gobies. All characteristics recorded were showed significant differences except for frequency(C). "Male Number" represents the different identities of each individual fish.

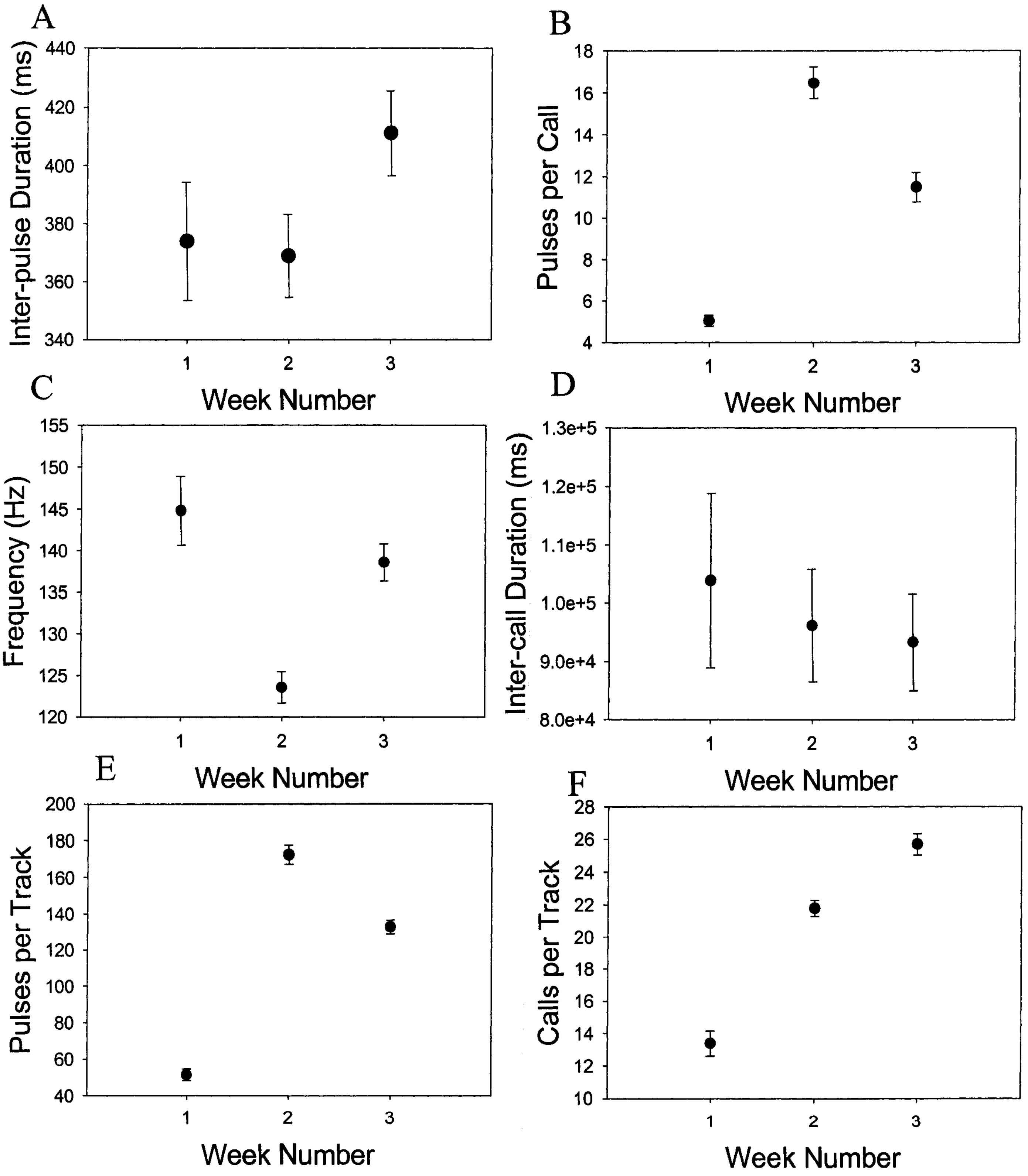

Figure 17: There was a significant effect of week post injection only when looking at the number of pulses per track (E) and the number of calls per track (F).

There was a significant effect of individual identity on the inter-call duration ($n = 4$, $df = 3$, $F = 10.595$, $p < 0.001$). A *post-hoc* Bonferroni test showed significant individual effects between fishes 1 and 2, fishes 1 and 3, and fishes 3 and 6 (Figure 16D). There were no hormone effects between weeks ($n = 3$, $df = 2$, $F = 2.880$, $p = 0.057$) (Figure 17D), and there was a significant interaction factor between male number and week number ($n = 7$, $df = 6$, $F = 3.532$, $p = 0.002$).

Pulses per 30 minute track showed significance for individual effects ($n = 4$, $df = 3$, $F = 79.127$, $p < 0.001$). A *post-hoc* Bonferroni test showed significant individual effects between fishes 1 and 2, fishes 1 and 3, fishes 2 and 6 and fishes 3 and 6 (Figure 16E). There were also hormone effects between weeks ($n = 3$, $df = 2$, $F = 24.573$, $p < 0.001$) (Figure 17E) and there was a significant interaction effect ($n = 7$, $df = 6$, $F = 185.183$, $p < 0.001$).

Finally, the analysis of the number of calls per 30 minute track also showed significant results for individual effects ($n = 4$, $df = 3$, $F = 91.113$, $p < 0.001$). A *post-hoc* Bonferroni test showed significant individual effects between fishes 1 and 2, fishes 1 and 3, fishes 2 and 3, fishes 2 and 6, and fishes 3 and 6 (Figure 16F). There was a significant hormone effect ($n = 3$, $df = 2$, $F = 10.142$, $p < 0.001$) (Figure 17F), and there were interaction effects between male number and week number ($n = 7$, $df = 6$, $F = 108.912$, $p < 0.001$).

Discussion

Due to the low sample size of this study it would not be wise to make any definite conclusions based solely on this work. However, there are many interesting trends that are revealed by this work that warrant further investigation.

The current study shows that in addition to individual variation in call structure existing, general trends indicate that the differences could be correlated with size. The larger males (fish number 3: 39.68 g, 132 mm TL and fish number 2: 24.26 g, 128 mm TL) produced statistically more pulses per call (Figure 16B), more pulses per track (Figure 16E), and more total calls per track (Figure 16F). The larger fish inter-call durations (Figure 16D) that were shorter in comparison to the smaller fish (fish number 1: 14.21g, 105mm TL and fish number 6: 13.78g, 110mm TL). So overall, these larger males are more vocally active, with more pulses both per call and per track as well as a shorter amount of time between calls.

Body size is an important variable affecting the reproductive success of males in many fishes that has been well documented in many studies (Perrone 1978, Noonan 1983, McKaye 1986, Myrberg et al 1986, Hert 1990, Wooton 1990, Bisazza and Marin 1991). If more replicates revealed that larger males are more vocally active this would be consistent with a study conducted by Takemura (1984) which found that larger fish emitted calls more frequently than the smaller ones (depending on the dominance-subordinance hierarchy) and that the power of the calls increased with increasing male size. In a study conducted by Lobel and Mann (1995), their analyses showed that the number of pulses, call duration, and pulse period increased with increasing size.

Unlike the other variables, there was no significant difference in the frequency of the calls between individuals. However the general trend showed that the higher frequency calls were produced by the largest fish (39.68g, 132mm TL). There is some evidence that among sonic teleosts larger fish produce lower frequency sounds than smaller fish (Myrberg et al. 1965, Fine et al. 1977, Rowland 1978, Myrberg and Riggio 1985). It is possible that this inconsistency between studies could be cleared up if the mechanism of sound production was known in this fish, which it is currently not. Depending on the mechanism, larger fish could have a physical advantage that allows for them to make calls with a slightly higher frequency.

Due to the investigative nature of this study we did not do any testing for the presence of increased hormones in the males post-injection. We based our analysis of the effect of hormone levels on call structure on the assumption that the injections caused the males to have an increased level of hormones and that these levels would decrease as time post-injection increased. However there seems to be a peak in activity during week 2 as there were increases in the number of pulses produced per call (Figure 17 B), an increase in the number of pulses present per track (Figure 17E), and the total number of calls present in a 30 minute track (Figure 17F). Also, inter-call durations (Figure 17D) and inter-pulse durations (Figure 17A) were lower in week 2 which could also be an indication of increased vocalization rate. This may be explained by the fact that we used LHRH to stimulate the reproductive system of the goby. LHRH is naturally made by the hypothalamus and then sent to the anterior pituitary gland. This gland is then stimulated to produce gonadatropin hormones which in turn act on the reproductive organs. This indirect path could result in the hormones not taking full effect when the first acoustic recording took place the following morning. Further research such as actual tissue sampling is needed to investigate this hypothesis. If further tissue sampling revealed an increase of vocal activity with an increase in hormones these results would be consistent with the few other studies done in this area. Johnston and Johnson (2000b) found that vocalizations were not observed in *E. crossopterum* when the fishes were reproductively inactive. Similar results were found in *Pimephales notatus* which did not produce sounds outside of their reproductive season and most sounds were produced in response to an aggressive encounter, whether with another male or a female (Johnston & Johnson 2000a).

Overall, acoustic communication is essential to various life processes of fishes. It has been studied in the past and currently is being studied extensively to better understand role of these vocalizations. This investigative study brings to light the fact that there is still much we do not know about fish vocalizations and the physiological mechanisms which regulate them as well as the importance of such vocalizations. However, the current research suggests the importance of temporal variations.

APPENDIX B

Antibiotics Protocol

Gentamycin Protocol (To ablate canal neuromasts) (Modified from Song et al. 1995)

- Add 0.02 g of gentamycin sulfate for every 1 L of dechloronated water (The current research used 12L at a time so 0.24 g of gentamycin sulfate was used each time)
- Submerge treatment fish into gentamycin solution with an air-stone (only set air-stone on low so you do not damage neuromasts)
- Remove fish after 24 hours, using a modified net to avoid unwanted damage to neuromasts

Streptomycin Protocol (To ablate Superficial and Canal Neuromasts) (modified from Montgomery et al. 1997)

- Add 0.5 g of streptomycin sulfate for every 1 L of dechloronated water (The current research used 12L at a time so 6 g of streptomycin sulfate was used each time)
- Submerge treatment fish into streptomycin solution with an air-stone (only set air-stone on low so you do not damage neuromasts)
- Remove fish after 3 hours, using a modified net to avoid unwanted damage to neuromasts

APPENDIX C

Phalloidin Staining Protocol

(Modified from Higgs et al 2003)

- Saccule epitheliums are dissected out of round goby heads that have been fixed in 4% parafelmeldehyde for 24 hours and placed in a 6-well plate with 200 μL phosphate buffer.
- Remove the phosphate buffer is carefully with a p-200 pippetteman
- Add 12.5 μL phalloidin (Oregon Green, Molecular Probes, Oregon USA) to each well.
- Let the epithelia stain for 20 mins
- Wet mount on slide with Phosphate Buffer and coverslip
- Look at slide under a fluorescent microscope (eg. Zeiss Axioskop 2 fluorescent microscope) under a magnification of 10x and 20x.

NOTE: Phalloidin is light sensitive so all steps in which phalliodin is used should be done in the dark

APPENDIX D

LHRH Injection Protocol

(Modified from a protocol obtained from Lynda Corkum, University of Windsor)

- Add 1 mg into 1.0 mL (H20) sterilized (1 mg is equalivalent to 1 container from Sigma)
- Add 0.1 mL of this solution into 10 microcentrifuge tubes
- Add 9.9mL of sterile H20 into each test tube
- Inject with 0.1mg/50g of fish next to the dorsal fin into the muscle.
- Keep the fish separate from other fish
- Repeat the injections every 4 hours, two more times (3 times total)

Sigma Product Number: L4513,
Name: DES-GLY10(D-ALA6) Luteinizing hormone releasing hormone ethylamide

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