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Experimental Analysis of Fish Sensory Systems: From Behavioural Responses to Physiological Extremes

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Experimental analysis of fish sensory systems: from behavioural responses to physiological extremes

By

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Experimental analysis of fish sensory systems: from behavioural responses to physiological extremes

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November 3, 2021

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ABSTRACT

Fishes are surrounded by various types of stimuli in their habitats which provide important information about their environments. Here, I investigated how various types of stimuli can affect the behaviour and physiology of two freshwater species: the invasive round goby (*Neogobius melanostomus*) and the black bullhead (*Ameiurus melas*). In chapter 2, I tested for a synergistic response in the round goby to multimodal stimuli, by presenting acoustic, visual, and olfactory stimuli separately and simultaneously. The results showed a significant decrease in the average respiration rate during multimodal stimuli suggesting that a synergistic response to multimodal stimuli occurred. The development of multimodal traps may be useful to control round goby populations in non-native habitats since multimodal signalling may be more attractive than unimodal signalling. Chapter 3 exposed black bullhead (*Ameiurus melas)* to various sound intensities (160, 165, 170 and 175 dB re 1 μPa) of boat noise to investigate regeneration of ciliary bundles following noise exposure. Black Bullhead exposed to 170 and 175 dB re 1 μPa of noise had a decrease in ciliary bundle counts but regenerated within 48 hours (Experiment 1) and 72 hours (Experiment 2). Ciliary bundle counts never reached control levels following exposure of 175 dB re 1 μPa of boat noise. Anthropogenic noise can cause immediate damage to auditory epithelium, but fish can quickly recover, giving hope to mitigation efforts for development of a quiet refuge.

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CHAPTER 1

MULTISENSORY INTEGRATION

Fish Communication

Abiotic and biotic cues within underwater habitats can aid in the navigation of fishes in their environment and allows them to respond to and localize important biological signals (Huijbers et al., 2012; Frommen, 2020). Fishes have evolved a diversity of sensory modalities to communicate via these signalling pathways. Fish communication can encompass visual, acoustic, and olfactory signals as well as electrocommunication and mechanosensory communication (Rosenthal, 2007; Burnard et al., 2008; Crampton, 2019; Popper & Hawkins, 2019). While electrocommunication and mechanosensory communication are undoubtedly important (Alves-Gomes, 2001; Bleckmann & Zelick, 2009; Kramer, 2012; Crampton, 2019), the purpose of the current review is to focus on visual, acoustic, and olfactory communication as they are much more common across the Teleostei.

Visual Communication

Fishes can produce and respond to visual signals and displays to communicate important information during reproductive events and agonistic interactions. During courtship, many species can use visual cues to attract mates and induce spawning (Castro et al., 2009; Smith & van Staaden, 2009). Agonistic encounters may involve aggressive displays, which are most common during breeding season where protection of offspring is vital to reproductive success (Desjardins et al., 2012; Forsatkar et al., 2017). Many species use colour, patterns, and pigments as visual signals for mate selection to attract mates and advertise the quality of an individual or for competition among conspecifics (Price et al., 2008). Guppies (*Poecilia reticulata*), swordtails (*Xiphophorus helleri*), and sticklebacks

(*Gasterosteus aculeatus)* are among the most well-studied species in terms of visual signalling and have been used as models to understand visual communication in fish (Rosenthal & Evans, 1998; Earley & Dugatkin, 2002; Grether et al., 1999; Kemp et al., 2008; Wright et al., 2016). Reproductive male threespine sticklebacks (*Gasterosteus aculeatus*) have a red colouration on the throat that is an important trait for females to assess during mate selection (Sparkes et al., 2008; Wright et al., 2016). Spine displays have also been shown to occur during aggressive interactions among male sticklebacks (Wright et al., 2016). Male green swordtails (*Xiphophorus helleri)* have a distinct ornamentation where the rays on the caudal fin are elongated making it look like a "sword" and is used for mate attraction and mate assessment (Rosenthal et al., 1996). Rosenthal & Evans (1998) investigated the role of ornamental tails during mate attraction by manipulating playback videos of male green swordtail exhibiting courtship behaviours and digitally removing their ornamental tail. They determined that females showed preference to intact males over edited males. Another species that has been extensively studied for its aggressive and territorial displays when confronting conspecifics is the male Siamese fighting fish (*Betta splendens*) (Peake et al., 2006; Forsatkar et al., 2017; Iwata et al., 2021). Both male and female Siamese fighting fish use gill flaring and fin spreading as visual displays in aggressive interactions (Forsatkar et al., 2017). While visual displays are clearly important for many fish species, the efficacy of using visual signals in aquatic environments may be limited by the light intensity and its spectral composition (Van der Sluijs et al., 2011). If the environment is too dim to transmit a visual signal, it may not be detected by the receiver or at least partially detected which may not convey the complete message the signaller intended (Van der Sluijs et al., 2011). Increases in turbidity in aquatic environments, as a result from eutrophication or sedimentary inputs, can cause a reduction in available light and can affect the strength of visual markers in species that engage in colour-mediated sexual selection (Seehausen et al., 1997; Wong & Candolin, 2007; Van der Sluijs et al., 2011).

Acoustic Communication

Many teleost fishes produce sound during courtship, spawning behaviours, agonistic and aggressive interactions, during feeding, in fright and in threat of a predatory attack (Kasumyan, 2009; Fine & Parmentier, 2015). In many fish species, vocalizations are a series of short low-frequency pulses that may repeat and be longer in duration (Ladich, 1997). Vocal teleost fishes have evolved various mechanism to produce sounds with the most common mechanism for sound production being the sonic swim bladder system and bone stridulation (Fine & Parmentier, 2015). The sonic muscles surrounding the swim bladder relax and contract to produce sound that is radiated in all directions (Parmentier et al., 2006; Fine & Parmentier, 2015). To produce sound via stridulation fish may use pharyngeal teeth, fins, rays, and vertebrae; by rubbing together skeletal structures they can produce rasps and creak noises that can propagate at a wide range of frequencies (Fine & Parmentier, 2015). Species within the toadfish family (Batrachoididae) like the Lusitanian toadfish (*Halobatrachus didactylus*) and the plainfin midshipman (*Porichthys notatus*) have been extensively studied for their sound production mechanisms and the role of sound during reproduction. The Lusitanian toadfish can emit long tonal boat whistle calls for male-male competition and attracting females to the nest for spawning (Amorim, 2006). The Lusitanian toadfish has a broad repertoire of sounds but only uses the boat whistle calls during mating season (Amorim, 2006) and produces them by rapid contraction of the sonic muscles that surround the swim bladder (Dos Santos et al., 2000; Amorim, 2006). The plainfin midshipman emits long uninterrupted humming sounds produced by the contraction of sonic muscles as well, which attracts females during breeding season (Ibara et al., 1983; Sisneros, 2009). Fish from the Sciaenidae (i.e., freshwater drum, *Aplodinotus grunniens*, atlantic croaker, *Micropogonias undulatus*) and Gobiidae (i.e., painted goby (*Pomatoschistus pictus)*, round goby (*Neogobius melanostomus*)) family of fishes have also been studied for their vocalizations (Ramcharitar et al., 2006; Amorim & Neves, 2008; Amorim et al., 2013a; Amorim et al., 2013b; Horvatić et al., 2019). The Gobiidae fishes for example, can produce drumming, stridulations, and tonal sounds during agonistic and reproductive contexts, but certain goby species lack a swim bladder and may produce sounds by the contraction of muscles that insert in the pelvic girdles, but the exact mechanism has not been determined (Lugli et al., 1996; Parmentier et al., 2013). Production of sounds is an energetic investment for vocal fish and enables them to communicate important information such as the quality of a mate which plays an important role in mate selection (Amorim et al., 2013b; Amorim et al., 2015).

Acoustic signals are effective because they can travel long distances regardless of light levels and water clarity. Sounds underwater propagate more efficiently than in air since sound in water travels 4.8 times faster (1500 m/s v. 343m/s). All fishes detect sound as particle motion which describes the magnitude, temporal and frequency characteristics and the direction of the travelling sound; enabling fish to localize, interpret, and respond to acoustic signals (Popper & Hawkins, 2019). Hearing capabilities in fish can vary among species as some contain specialized hearing structures that enable them to detect a broad range of frequencies (several kHz). Perches, sunfishes, and salmonid species without enhanced hearing can detect sounds at <1kHz (Fine & Parmentier, 2015). The Weberian apparatus is a specialized hearing structure which is composed of a series of bones that connects the swim bladder to the inner ear of the fish. This structure contributes to the enhanced hearing in the superorder Ostariophysi, which include species of minnows, carps (Cypriniformes), and catfishes (Siluriformes). Although enhanced hearing capabilities has its advantages on survival, by being more sensitive to abiotic and biotic sounds, it also means that fishes with enhanced hearing can be more affected by high intensity sounds, specifically anthropogenic noises resulting from human activities near or in aquatic environments (boat noise, seismic exploration, coastal urban developments) (Radford et al., 2014; Popper & Hawkins, 2019).

Olfactory Communication

In olfactory signalling, chemical stimuli are detected by fishes which activates the olfactory receptors and transmits signals to the brain so fish can respond appropriately (Sorensen & Stacey, 2004; Touhara, 2013). Olfactory signals can convey important information regarding reproduction, species recognition and aggregation, migration, schooling/shoaling, and predatory avoidance (Sorensen & Stacey, 2004; McCormick & Manassa, 2008; Touhara, 2013). Olfactory communication is an important sensory modality as it provides chemical information about the sensory scene. Pheromones, which are odours or odour mixtures composed of soluble steroids, bile acids, and proteins are released by a fish (sender) to a conspecific (receiver) and convey significant information that can induce species-specific behavioural responses (Burnard et al., 2008; Touhara, 2013). Pheromones can act as releasers or primers. Releaser pheromones induce immediate behavioural responses and primer pheromones can induce developmental and/or endocrinological responses which make take longer to materialize (Touhara, 2013). The use of pheromones in low light environments is a great advantage to fishes as they are still able signal to each other and communicate regardless of light levels (Sorensen & Stacey, 2004). Sex pheromones released by fishes during reproduction play an important role in mate attraction and successful reproduction. Even before male and female fish are sexually mature, they can respond to pheromones by increasing gonadal development or hormonal changes that induce finale gamete maturation (primer) and once they are sexually mature, they may respond by exhibiting spawning behaviours (releaser) (Sorensen & Stacey, 2004; Touhara, 2013). In species aggregation, pheromones can aid in recognizing conspecifics or offspring and aid in schooling/shoaling and migration behaviours (i.e., salmonids) (Sorensen & Stacey, 2004; Touhara, 2013; Bett & Hinch, 2016). Alarm signals in fish can play a role in predator avoidance. The alarm cues are released from club cells found on the skin when fish have been hurt or recently eaten which signals that there is a threat present to surrounding fish species (Magurran & Irvin, 1996; Sorensen & Stacey, 2004). Research involving alarm cues shows that responses to alarm cues can vary among species as well (Magurran & Irvin, 1996; Wisenden, 2008; Sorensen & Stacey, 2004). Although there is still some debate on whether alarm cues are considered true pheromones, they still play an important role in predator avoidance in many species.

Pheromones can be used to control invasive fish populations by trapping, disrupting migration and movement, and repelling fish from certain ecosystems (Sorensen & Stacey, 2004). The benefit of using pheromones as a control for invasive population is that it is species-specific and will not affect non-target species (Ochs et al., 2013). This method has been successful in controlling populations of sea lamprey (*Petromyzon marinus*), an invasive species in the Laurentian Great Lakes (Imre et al., 2010). The use of predator cues can repel sea lamprey from species habitats (Di Ricco et al., 2016) and male pheromones can attract females into baited traps (Johnson et al., 2009; Johnson et al., 2015; Dawson et al., 2016). Pheromones overall have a significant role in life histories of fish and mediate many behavioural responses that are crucial to fish survival and their reproductive success.

Unimodal and Multimodal Signalling

Unimodal and multimodal signalling in fishes undoubtedly plays an important role in a fish's behaviour and life history. Presenting various types of unimodal and multimodal stimuli to fishes can elicit specific behavioural responses that aid in understanding how fishes can perceive and interact with their environment (Hebets & Papaj, 2005; Partan & Marler, 2005). Multimodal communication, which can be categorized as redundant or nonredundant, involves producing and receiving signals through more than one sensory channel which simultaneously communicates multiple elements of an animal's behaviour (Partan & Marler, 1999; Partan & Marler, 2005; Kasurak et al., 2012). Behavioural responses to multiple redundant signals can result in a) where the intensity of a behavioural response is equal to behavioural responses to individual redundant signals in isolation (equivalence) or b) where the intensity of a behavioural response is much greater than the response to individual redundant signals in isolation (enhancement or synergistic) (Partan & Marler, 2005; Kasurak et al., 2012). Multiple nonredundant signals can result in a) each signal having an independent effect (independence) or b) where one signals can affect or change the other signals (dominance) or c) where a completely new response occurs when multiple signals are used in combination (Partan & Marler, 2005). Multimodal signalling can improve localization and detection of a signal where one channel can be used to attract and the other to convey the intended message (Partan & Marler, 2005; Kasurak et al., 2012). There is a high energetic cost when producing and receiving multimodal signals. Multimodal signalling increases the risk of predation since senders become more conspicuous in their environment because they are emitting multiple signals simultaneously (Partan & Marler, 2005). The receivers of multimodal signals tend to invest more time in signal detection than predator detection which can ultimately affect their survival (Partan & Marler, 2005). Despite the disadvantages, there are great benefits in using multimodal signalling as it increases the accuracy of signals ensuring that the intended message is not distorted and that the true intended signal is received correctly. Communicating via multiple sensory modalities can compensate for different abiotic and biotic factors that may limit certain signals and ensure that the receiver can localize and respond appropriately to biologically relevant signals (Partan & Marler, 2005).

Visual Experiments

Visual signalling experiments have used different types of stimuli to elicit behavioural responses in fishes and have accomplished this by using mirrors, live stimuli, dummies, and video playback (Rowland, 1999). Using different types of stimuli can aid in understanding intraspecific and interspecific interactions and determine the role and importance of visual displays during reproduction and agonistic encounters. Using mirrors as visual stimuli has been done quite extensively to study aggressive displays (Elcoro et al., 2008; Desjardins & Fernald, 2010; Balzarini, et al 2014) and lateralization in fishes (Cantalupo et al., 1996; Sovrano et al., 2001; Takeuchi et al., 2010). Siamese fighting fish (*Betta splendens*) have often been used to study aggression. Mirror images that are used in these experiments are considered a “conspecific” despite being an exact duplicate of the study subject and therefore may result in a different type of response than when using a live stimulus. (Rowland, 1999; Desjardins & Fernald, 2010; Cattelan et al., 2017). The benefit of using a mirror image is that it can standardize and control the stimulus (if one needs an unmodified stimulus) and can be effective when used in the proper context (Balzarini et al., 2014).

An alternative method to mirror images is using live stimuli. Using live fish as stimuli in behavioural experiments allows the study subject to produce behavioural responses that would typically occur in nature when interacting with a conspecific rather than a mirror image stimulus, which would not occur in nature. Live stimuli studies can explore courtship and reproductive events elucidating what visual cues are at play in mate attraction (Smith & van Staaden, 2009; Hughes et al., 2013). For example, Smith & van Staaden (2009) revealed that Malawian cichlids (*Metriaclima spp.; Melanochromis spp.*) use both multimodal and unimodal signalling equally (acoustic calls and visual displays) during courtship and that the courtship strategies within the species can vary. Presenting multimodal stimuli via a live stimulus has shown to be more attractive than unimodal stimuli in fishes where combining sensory modalities can attract mates to induce spawning (Amorim et al., 2013a). Experiments of this nature can also study agonistic encounters and observe these behaviours at a closer magnitude. The role of colour and markings on fish are important in mate attraction and agonistic interactions where colour and markings can work to attract mates or warm off predators or competitors (Price et al., 2008; John et al., 2021). The limitations of using live fish as stimuli are that they cannot be controlled like that of a mirror image. The live stimulus can therefore interact and engage in behaviour that may influence the behaviour of the study subject and as a result can affect the outcomes of these types of experiments.

Using realistic models of fishes allows researchers to have better control over this type of visual stimulus (Rowland, 1999). Social interactions among fishes can be investigated by using realistic models to control for certain aspects of an interaction. Dzieweczynski et al. (2009) used a model of a female Siamese fighting fish (*Betta splendens*) to elicit courtship responses in a male Siamese fighting fish while in the presence of a male conspecific and showed that courtship and nest monitoring increased in the presence of another male. Realistic models can also be used in experiments that investigate competitor assessment in agonistic interactions. Yavno & Corkum (2010) presented female round gobies to a combination of olfactory cues and visual models of male round gobies and determined that females showed preference to visual male models over olfactory cues. Beeching et al. (1998) studied the sexual dichromatism in female convict cichlids (*Amatitlania nigrofasciata*) by presenting live fish and realistic models showing preferences over bright ventral colouration of live conspecifics and realistic models. Females also exhibited increased aggression towards the brightly coloured stimuli showing preference to bright ventral colouration over intermediate ventral colouration. Realistic models allow for better control and manipulation of certain morphological structures that can influence behavioural responses in a study subject. Visual characteristics and structures such as sexual ornaments or colour and markings can be altered or removed with the aim in determining the importance of these characteristics in mate selection (Rowland, 1999). A limitation to using this method is that they may not be as effective at eliciting responses from fish as the target fish may not recognize the model as a conspecific due to lack of other cues (i.e., olfactory, and acoustic cues).

The video playback method has been used by researchers as a visual stimulus in experiments. With this method researchers have more control over what types of behaviours are presented to the study subject as they can isolate specific behaviours and digitally enhance or erase certain visual characteristics (i.e., sexual ornaments, colours) on fishes (Rosenthal et al., 1996; Rosenthal & Evans, 1998; Rowland, 1999). Using video playback alone and in combination with other types of visual stimuli can allow researchers to study species recognition (Balshine & Lotem, 1998) and mate choice and assessments (Robinson-Wolrath, 2006) through quick presentation and efficient modification of visual characteristics (Rosenthal & Evans, 1998). Some disadvantages to using this method are that the devices used are created for human perception and what humans see (Rowland, 1999) and therefore if the spectral sensitivity of a fish is different than that of humans, they may not see the playback as a continuous video and rather, perceive it as discontinuous. Although the previous methods have their disadvantages, they are a still valid methods to use in visual experiments and can answer important scientific questions about fish behaviour if applied appropriately (Rowland, 1999).

Acoustic Experiments

Acoustic playback experiments have explored how fish can distinguish and localize sounds of conspecifics (mating calls) and determine the effects of anthropogenic noise on behaviour and physiology. For example, the plainfin midshipman, the Lusitanian toad fish and species in the Gobiidae family have been used to investigate behavioural responses to conspecific call (McKibben & Bass, 2001; Rollo et al., 2007; Rollo & Higgs, 2008; Zeddies et al., 2010; Vasconcelos et al., 2011). The use of playback experiments can reveal how call characteristics can advertise the condition of the male producing them and how it may be linked to body size, which was investigated in the painted goby (Amorim et al., 2013a; Amorim et al., 2013b). Acoustic playback experiments involving the round goby have shown that they can localize and respond to conspecific calls and that reproductive state can also influence how they respond to acoustic signals (Rollo et al., 2007; Isabella-Valenzi & Higgs, 2013) outlining the importance of acoustic signalling in reproduction. The plainfin midshipman and the Lusitanian toadfish also show abilities of localizing and responding to calls (Zeddies et al., 2012) and advertise quality when calling to females (Amorim et al., 2010; Zedies et al., 2010). Acoustic experiments have also exposed fishes to anthropogenic noise through playback experiments. Studies of this nature can investigate negative effects anthropogenic noise which has been shown to cause increases in stress hormones, increases in ventilation rage, damage to the inner ear and swim bladder rupture and can disrupt normal behaviour such as foraging, predator and prey interactions, and startle and sheltering responses (Purser & Radford, 2011; Sabet et al., 2016; Pieniazek et al., 2020). The acoustic playback method can be applied as a method to control populations of invasive species (Miehls et al., 2017; Bzonek et al., 2021; Heath et al., 2021) where acoustic traps can be set up in vulnerable habitats and be used to attract and trap invasive species and be removed (Isabella-Valenzi & Higgs, 2016). Conducting playback experiments using fish vocalizations has its drawbacks. Playing recording of calls in a tank in laboratory settings can alter the characteristics of fish vocalizations due to reverberation, which may unintentionally prolong or distort the sound being played (Akamatsu et al., 2002; Rogers et al., 2016).

Olfactory Experiments

Responses to olfactory stimuli can be used to identify putative reproductive pheromones, determine how fish can recognize and localizes conspecific odours, and understand the olfactory system overall (Sorensen & Stacey, 2004; Burnard et al., 2008). Olfactory experiments can present sex pheromones of conspecifics to observe what type of effects they have on behaviour (Sorensen & Stacey, 2004; Burnard et al., 2008; Kasurak et al., 2012). Fish can also be exposed to alarm cues to explore anti-predator responses (Wisenden, 2004; McCormick & Manassa, 2008; Wisenden, 2008). Reproductive status also has an influence on behavioural responses with reproductive morphs of various species are more attracted to pheromones of conspecifics than non-reproductive morphs (Kasurak et al., 2012). All-important putative pheromones have been identified in the common goldfish (*Carassius auratus*) and it is considered an important model in understanding olfactory signalling (Sorensen & Stacey, 2004). Pheromones can also be used to trap and repel invasive species from vulnerable habitats (Sorensen & Stacey, 2004; Luehring et al., 2011; Ochs et al., 2013). A disadvantage of using pheromones may be that the concentrations of pheromones used experiments may be too high and do not naturally occur in nature.

Integrating Signals

In recent years, studies involving multimodal signalling has started to increase. Previous studies involving presentation of stimuli to fish have used unimodal stimuli and multimodal stimuli to understand how fishes use this type of signalling and respond to multiple signals presented simultaneously (McCormick & Manassa, 2008; Smith & van

Staaden, 2009; Yavno & Corkum, 2010; Kasurak et al., 2012). While the use of unimodal signals in behavioural experiments is important, it is necessary to move towards an integrative approach to better understand multimodal signalling and fish behaviour in its entirety. For example, African cichlids species use multimodal signals (visual and acoustic cues) during courtship (Smith & van Staaden, 2009) and acoustic signals augment visual displays, but never occur on their own (Smith & van Staaden, 2009). Using a combination of cues can ensure reproductive success. Visual and olfactory cues are important for assessment in fish as visual and olfactory cues can be used assess predators and potential mates and yield a stronger antipredator response (McCormick & Manassa, 2008; Yavno & Corkum, 2010). Kasurak et al. (2012) used acoustic and olfactory stimuli in their study to show how integrating signals can lead to enhanced responses. By exposing reproductive and non-reproductive female round gobies to acoustic calls and male conditioned goby water they determined that reproductive females were more attractive and responsive to multimodal stimuli than non-reproductive females. Reproductive females also showed a more enhanced response to multimodal signalling. Integrating signals can lead to more enhanced responses that benefit survival and reproductive success. Multimodal signals can convey information more efficiently, since the time it takes for a fish to respond can be reduced and accurately assess the signaler and the information being communicated (Kasurak et al., 2012).

Sexual Maturity: Behavioural Responses and Production of Signals

Fish undergo physical and physiological changes with growth, such as maturation of reproductive organs, prior to reaching sexual maturity (Treasurer, 2021). Upon reaching sexual maturity, they begin to exhibit reproductive behaviours and can begin to spawn with potential mates (Lowerre-Barbieri et al., 2011; Treasurer, 2021). The sensory signals used in fish communication can include acoustic vocalizations and visual displays used during courtship and agonistic contexts (Amorim, 2006; Rollo & Higgs, 2008; Sisneros, 2009; Maruska et al., 2012; Earley & Dugatkin, 2002; Dzieweczynski et al., 2009) and the release of pheromones to attract conspecifics and induce spawning behaviours (Burnard et al., 2008). The reproductive status of a fish can influence how they produce and respond to sensory signals within their environment (Sisneros & Bass, 2003; Clement et al., 2005; McLennan, 2005; Sisneros, 2009).

Studies that investigate the behavioural responses to sensory modalities of a reproductive morph of a fish species to a non-reproductive morph can shed light on the role that reproductive maturity has on the responsiveness to reproductive signals (i.e., calls, visual displays, pheromones). Reproductive morphs of many species show higher preferences and sensitivities to reproductive signals than their non-reproductive counterparts (Sisneros & Bass, 2003; Clement et al., 2005; McLennan, 2005; Sisneros, 2009). During breeding season, the plainfin midshipman (*Porichthys notatos*) produces vocalizations to attract females and the onset of these vocalizations coincides with the increase in the range of frequency sensitivity in the inner ears of reproductive females so that they can detect and localize male calls (Sisneros, 2009). In a separate study, female reproductive plainfin midshipmans were able to discern important reproductive vocalizations up to 340 Hz while non-reproductive females can only perceive calls up to 100 Hz demonstrating that reproductive state can influence the responses to male vocalizations at a neurophysiological level (Sisneros & Bass, 2003). The reproductive state in female African cichlid fish (*Astatotilapia burtoni*) can influence affiliation and preference to territorial male conspecifics, with gravid females of this species showing preference to territorial males over non territorial while non-reproductive females showed no preference to either male type (Clement et al., 2005). Female brook sticklebacks *(Culaea inconstans)* are attracted to male olfactory cues depending on their reproductive maturity and participated in spawning behaviour (McLennan, 2005)

The invasive fish, the round goby (*Neogobius melanostomus*) uses sex pheromones to attract mates along with acoustic and visual signals. Previous studies have shown how behavioural responses in non-reproductive morphs differ from the reproductive morphs when presented with putative pheromones. Belanger et al. (2004) looked at the behavioural and electrophysiological responses of non-reproductive and reproductive females to water conditioned by a reproductive male round goby. Reproductive females spent more time swimming and near the stimulus zone and responded more to the conditioned water than the non-reproductive females. By measuring the olfactory epithelial field potential, they determined that the conditioned water used was potent enough to elicit a response from reproductive females (Belanger et al., 2004). Kasurak et al. (2012) showed similar results where they presented unimodal and multimodal signals (acoustic and olfactory cues) to non-reproductive and reproductive females and showed that reproductive females responded to not only unimodal signalling but showed preferences to multimodal signals unlike the non-reproductive females which showed weak responses or rarely responded at all. Given the findings of these studies, is it evident that reproductive status can influence behavioural responses to signals that are important for reproductive success.

Anthropogenic Noise

Over the last several decades, the presence of anthropogenic noise in aquatic habitats has increased across the globe and continues to negatively impact aquatic environments (Kunc & Schmidt, 2021). Anthropogenic noise is disruptive and unwanted sound that can be categorized as a) transient/impulsive sounds that are short in duration, are repetitive, and show large changes in amplitude over time (i.e., underwater explosions or seismic airguns) or b) continuous sounds which are present for a long period of time, from months up to years (i.e., large shipping vessels, recreational boats or wind turbines) (Hildebrand, 2004; Popper & Hawkins, 2019). Other sources of anthropogenic noise include urban developments in coastal areas, underwater resource extraction and seismic exploration devices (Popper, 2003; Radford et al., 2014; Mickle & Higgs, 2018; Popper & Hawkins, 2019). The impact of anthropogenic noise in marine environments has been extensively studied (Weilgart, 2007; Clarke et al., 2009; Popper et al., 2020), but there are still gaps in our knowledge on the full impacts noise pollution has on freshwater fishes (Popper, 2003; Slabbekoorn et al., 2010; Mickle & Higgs 2018; Popper & Hawkins, 2019). The most prevalent source of anthropogenic noise is shipping noise as it propagates efficiently at low frequencies underwater and may overlap with acoustic communication signals among vocal freshwater fish species (Richardson & Würsig 1997; Vasconcelos et al., 2007; Radford et al., 2014).

The physiology and behaviour of fishes can be affected when exposed to noise pollution (Mickle & Higgs, 2018). Physiological stressors can include increases in glucocorticoids (GC) (a stress indicator) such as cortisol, which have been observed in the goldfish (*Carassius auratus*) (Smith et al., 2004), the black tail shiner (*Cyprinella venusta*)

(Corvo et al., 2015) and in three European freshwater fishes, (common carp, *Cyprinus carpio*, the gudgeon, *Gobio gobio*, and the European perch (*Perca fluviatilis*) in response to anthropogenic noise (Wysocki et al., 2006). Other responses to physiological stressors can include increased ventilation rate (Purser et al., 2016) and acoustic trauma to the inner ear of the fish where high intensity sounds noises can cause damage to the auditory system and induce temporary or permanent threshold shifts resulting in loss of hearing (Scholik & Yan, 2001; McCauley et al., 2003; Smith et al., 2004; Smith et al., 2011). Swim bladder ruptures can also result from exposure to high intensity sounds (Casper et al., 2013). The presence of boat noise in aquatic environments can also disrupt natural activities and behaviours such foraging and foraging efficiency, predator and prey interactions, and startle and sheltering responses (Purser & Radford, 2011; Sabet et al., 2016; Pieniazek et al., 2020).

The ability to perceive and respond to sound is important for many fish species as there are over 800 species, from 109 families, known to produce vocalizations during agonistic and territorial encounters, during spawning and courtship behaviours (Amorim et al., 2015; Slabbekoorn et al., 2010; Popper & Hawkins, 2019). Anthropogenic noise may mask important vocalization and interfere with reproductive success in terms of responding to or assessing vocalizations of a potential mate (Codarin et al., 2009; Slabbekoorn et al., 2010; Popper & Hawkins, 2019). Anthropogenic noises can either fully mask the vocalization, where no information can be communicated, or partially mask it where some information is communicated but not enough for the receiver to respond appropriately (Clark, 2009; Radford et al., 2014). As previously mentioned, temporary or permanent auditory threshold shifts may occur when fish are exposed to high intensity sounds. Hearing loss can decrease the ability to fully assess the aquatic soundscape which includes being able to respond to ecologically relevant signals such as vocalizations that are produced during courtship. Vocalizations are an integral part of the reproductive tactics of many vocal species and aid in assessing and choosing potential mates (Amorim, 2006; Kasumyan, 2009). Hearing impairment can lead to poor predator and prey detection and difficulty in participating and responding to agonistic interaction, which can ultimately affect survival (Ladich, 2008; Sebastianutto et al., 2011; Simpson et al., 2016) The effects of anthropogenic noise on fish can vary greatly and cause detrimental effects however there is growing evidence that fish can regenerate sensory hair cells following noise exposure (Smith et al., 2004; Smith et al., 2006; Schuk & Smith, 2009; Scholik & Yan, 2001). It is important to note that this does not minimize the effect on hearing in fish specifically, but it does give us insight on how fish may be compensating for louder environments. The ability for fish to regenerate sensory hair cells may vary due to different hearing capabilities. For example, Scholik & Yan (2002) conducted a study of the effect of acoustic trauma on bluegill sunfish, a species with no enhanced hearing capabilities, and showed no significant effect on hearing. However, a previous study conducted by the same authors (Scholik & Yan, 2001), tested the effects on anthropogenic noise on the fathead minnow (*Pimephales promelas*), a cyprinid fish with enhanced hearing abilities, at the same intensity and showed that there was an effect on hearing; therefore, the effects of anthropogenic noise may be species dependent, where fish species with a broader bandwidth can experience more intense effects. Anthropogenic noise is a very serious stressor on the physiology and behaviour of fishes and will continue to be omnipresent in aquatic habitats due to the increase in globalization. Therefore, it is pertinent to continue to develop this field in effort to implement policies that will protect vulnerable aquatic animals.

The Round Goby (*Neogobius melanostomus*)

The round goby (*Neogobius melanostomus*) is a benthic teleost fish species that belongs to the Gobiidae family of vocalizing fish (Brown & Stepien, 2009) and is native to the Ponto-Caspian region which includes the Black, Capsian, Azov, and Aral Seas (Corkum et al., 2004). The round goby was introduced to the Baltic Sea, many major European Rivers, and the Laurentian Great Lakes in 1990 by the ballast water of ships originating from the Ponto-Caspian region (Jude et al., 1992; Charlebois et al., 2001; Hensler & Jude, 2007; Kornis et al., 2012). The round goby has successfully invaded and proliferated in these new habitats where it also outcompetes many native benthic species (Dubs & Corkum, 1996; Corkum et al., 2004; Kornis et al., 2012). In less than a decade, this invader had spread to all five Laurentian Great Lakes affecting benthic fish species such as the mottled sculpin (*Cottus bairdii*), the logperch (*Percina caprodes*), the northern madtom (*Noturus stigmosus*), the eastern sand darter (*Ammocrypta pellucida*), and many species of freshwater mussels (Dubs & Corkum, 1996; Corkum et al., 2004; Balshine et al., 2005; Nurkse et al., 2016). The success as an invader can be attributed to various factors; they have a high tolerance to varying environmental factors, have a broad diet, exhibit aggressive behaviours (chases, bites, and approaches) and are able to spawn repeatedly throughout the spring, summer, and autumn (Dubs & Corkum, 1996; Corkum, 2004; Kornis et al., 2012). Round gobies are often used as bait which further aids their expansion to new habitats, as well as the transfer of contaminants through the food web due their diet of benthic organisms (i.e., amphipods, crayfish, dreissenids and isopods) (Corkum et al., 2004). Round gobies can be preyed upon by larger sport and commercial fish resulting in bioaccumulation which can negatively impact human health (Corkum et al., 2004). Since invading the Great Lakes, the round goby has become an important food source for many native predator species such as smallmouth bass (*Micropterus dolomieu*), yellow perch (*Perca flavescens*), walleye (*Sander vitreus*), and the burbot (*Lota lota*) (Reyjol et al., 2010; Crane et al., 2015).

There are two male round goby morphs: Type I parental nest-guarding males and Type II sneaker males (Corkum et al., 1998; Marentette et al., 2009). Type I males are large and are black in colouration (body and fins), have enlarged cheeks and an enlarged urogenital papilla, located on the ventral side of the fish between the anus and base of anal fin (Corkum et al., 1998; Kornis et al., 2012). Reproductive females have an enlarged urogenital papilla that changes from white to orange in colour (Kornis et al., 2012; Donovan, 2015). Type II males or sneaker males have enlarged testes and urogenital papillae (Corkum et al., 1998) and are smaller than Type I males, as they invest in reproduction rather than overall growth (Marentette et al., 2009). Sneaker males are a mottled brown colour like non-reproductive morphs and lack secondary characteristics (black colouration and puffy cheeks) (Corkum et al., 1998; Marentette et al., 2009). During reproduction, sneaker males stealthily and quickly add their ejaculate during spawning events between Type I males and reproductive females. They may also imitate females to successfully fertilize a female's egg during sperm competition (Marentette et al., 2009). In preparation for spawning, the Type I males create nests underneath an overhanging hard substrate and fans the nest with their pectoral and caudal fins before egg deposition (up to 10 days before) while also releasing important reproductive pheromones (3α-hydroxy-5βandrostane-11,17- dione-3-sulfate (11-O-ETIO-3-S) and 3α,17β-hydroxy-5β-androstan11-one-17-sulfate (17-S)) through their urine to attract reproductive females (Belanger et al., 2004; Corkum et al., 2006; Meunier, 2009; Farwell et al., 2017). Nest-guarding males have been reported to show increased ventilation to urogenital tissue extracts and the female putative pheromone estrone indicating the importance of olfactory cues for mate attraction and successful spawning (Corkum, 2004). To further prepare the nest, the male rubs secretions on the nest ceiling and once the nest is ready, it uses visual, olfactory, and acoustic cues to attract and lure the female to the nest (Corkum et al., 1998). Once inside the nest the females invert themselves and lay their eggs on the nest ceiling and the male then spreads sperm on the nest ceilings to fertilize the eggs (Meunier et al., 2009; Kornis et al., 2012). After spawning occurs, the nest is guarded by the male until the eggs hatch and the male exhibits a fanning behaviour to provide oxygen to the eggs using its caudal and pectoral fins (Wantola et al., 2013; Kornis et al., 2012). While guarding, the male may exhibit aggressive behaviour such as raising pectoral and dorsal fins, emitting vocalizations, and chasing and attacking the intruder (Meunier et al., 2009; Kornis et al., 2012). Aside from agonistic contexts, vocalizations have also been observed during and after egg deposition and as a tactic for mate attraction (Rollo et al., 2007)

Reproductive male round gobies use vocalizations in combination with visual and olfactory cues to attract females to the nest to induce spawning (Rollo et al., 2007; Isabella-Valenzi & Higgs, 2013). Yavno & Corkum (2009) showed that reproductive females were more attracted to visual models at a nest rather than the reproductive male. However, it is important to note that round gobies use many sensory modalities to attract their mates for successful reproduction, with certain modalities being favoured over the other depending on their environment (Kasurak et al., 2012). Round gobies can produce auditory signals through a series of low frequency pulses (dominant energy ~180Hz) (Zeyl et al., 2013; Isabella-Valenzi & Higgs, 2016) for mate attraction and agonistic displays. The exact mechanism for vocalizations is not known for this species but previous studies have successfully recorded vocalizations and used them in playback experiments to understand mate attraction and localization of calls (Rollo et al., 2007; Rollo & Higgs, 2008). Regarding hearing, round gobies do not have enhanced hearing capabilities as they have no accessory hearing structures and lack a swim bladder (Zeyl et al., 2013). Round gobies can hear from 100 Hz- 600 Hz and have an auditory threshold of 145-160 dB re 1 μPa (Belanger et al., 2010).

Acoustic studies involve investigating sound production and the attraction of localizations of round gobies to conspecific calls through playback experiments which reveal that although the mechanism is not entirely known they are able to localize and recognize and differentiate between calls (Rollo et al., 2007; Rollo & Higgs, 2008; Isabella-Valenzi & Higgs, 2013). Wagner et al. (2015) investigated the effects of seismic water guns on the inner ears of the round goby and determined that their ears were not affected indicating that perhaps higher sound intensities are needed to damage sensory epithelium. Round gobies do not have enhanced hearing capabilities or accessory hearing structures (Weberian ossicles or swim bladder) which may contribute to their reduced hearing sensitivity and require much louder sounds to cause morphological and physiological damage to the inner ear (Wagner et al., 2015). Additionally, exposure to moderate anthropogenic noise (>140 dB re 1 μPa) has little effect on calling rate (Higgs & Humphrey, 2020). Anthropogenic noise does not seem to impact round gobies like other species with hearing specializations, for example, and can therefore make the round goby more resistant to inner ear damage caused by anthropogenic noises and further add to its success as an invasive species.

The sensory studies done on the round goby have also focused on investigating olfactory signalling and the role of pheromones during reproduction. Olfactory studies have explored how female and male gobies respond to sex pheromones (Murphy et al., 2001; Belanger et al., 2004; Gammon et al., 2005; Marentette & Corkum, 2008; Laframboise et al., 2011) and other odours (Sreedharan et al., 2009). Round gobies release a sex pheromone that activates spawning behaviour in reproductive females. Electro-olfactogram (EOG) assays can measure the olfactory epithelial field potential in response to water conditioned by reproductive males (Belanger et al., 2004). Conditioned water is a strong olfactory stimulus in reproductive females showing that reproductive status may influence the type of behavioural response of these sex pheromones (Belanger et al., 2004; Kasurak et al., 2012). Reproductive females often show spawning activity induced by olfactory stimuli while non reproductive females do not show these behaviours when exposed to male conditioned water. Reproductive male round gobies use sex pheromones to attract mates indicating that pheromones are a crucial part of their reproductive tactics (Zielinski et al., 2003; Tierney et al., 2013). The use of pheromones is quite beneficial since olfactory signals can still be received by other conspecifics regardless of light levels and clarity. Round goby pheromones are also species-specific as native species such as rock bass (*Ambloplites rupestris*), blue gill sunfish (*Lepomis machrochirus*), pumpkinseed sunfish (*Lepomis gibbosus*), smallmouth bass (*Micropterus dolomieu*) and yellow perch (*Perca flavescens*) do not respond to putative pheromones and are unaffected by goby pheromone traps (Ochs et al., 2013). The morphology of the olfactory chamber shows that olfactory sensory neurons (OSN) are widely distributed throughout the olfactory epithelia and contained a single olfactory lamella accessory sac that indicate that this species can regulate odorant flow and may be driven by gill ventilation (Belanger et al., 2003; Belanger et al., 2006).

Visual stimuli also play an important role in round goby interactions. Presenting male and female round gobies to fibreglass models of conspecific intruders of varying body size and colouration can influence how soon male and females exited their shelters, with males and females exiting shelters sooner when presented with smaller male models than with large male models (Speares et al., 2007). Colouration of all types of models did not show a significant effect, despite the black colouration during breeding season, this characteristic may not be the one and only characteristic assessed during courtship by female, as gobies can emit calls and release pheromones to attract mates (Speares et al., 2007). Using a combination of calls, pheromones, and visual signals may help to attract and trap round gobies in the effort of controlling their growing population and spread in non-native habitats (Isabella-Valenzi & Higgs, 2016).

The Black Bullhead (*Ameiurus melas*)

The black bullhead (*Ameiurus melas*) is a demersal freshwater fish that belongs to the Ictaluridae family of fishes and is native to North America and Mexico (Cucherousset et al., 2008; Kreutzenberger et al., 2008; Rutkayová et al., 2008). The Black bullhead also belong to the order Siluriformes, which is known to have species with enhanced hearing capabilities due to the presence of the Weberian apparatus, an accessory hearing structure that comprises of a series of ossicles that connect the swim bladder to the inner ear of the fish (Chardon et al., 2008; Mickle et al., 2018; Pieniazek et al., 2020). Black bullheads inhabit warm waters and are usually found in small low-gradient streams, ponds, and backwaters of rivers, where they prefer soft bottomed substrates (Scott & Crossmain 1973; Hasnain et al., 2010; Copp et al., 2016). Tolerant to harsh conditions, bullhead can survive in environments with low dissolved oxygen (3.0 mg L-1), high water temperatures, up to 35˚C, and pollutants (Scott and Crossman 1973; Stuber 1982; Novomeská & Kováč, 2009; Copp et al., 2016). Considered a benthivorous and detritivirous feeder, black bullhead can consume a wide variety of prey items within their habitats as well as live fishes (Kreutzenberger et al., 2008). This species is considered a generalist species and usually feeds on the most abundant prey within their habitats (Leunda et al. 2008; Copp et al., 2016).

The reproductive and spawning strategies of the black bullhead have been reported by Wallace (1967) where reproductive behaviour was observed in captive black bullheads. The reproductive process of this species begins by the female excavating a nesting area in the substrate to prepare an area to deposit eggs. Once the nest is created, both male and female black bullhead embark on a courtship ritual that involve swimming near each other and touching each other with their barbells (Wallace, 1967). The pair participate in an "embrace" where the male uses his caudal fin to hold the female's head in place and repeats this behaviour until the female deposits her eggs in the nest. The female fans and guards the eggs the first day after egg deposition. The male then takes over in guarding the nest (Wallace, 1967). Black bullhead reach sexual maturity after 2 or 3 years of age and are considered spring spawners as they prefer to spawn once water temperatures reach 21˚C (Jenkins and Burkhead, 1993; Novomeská & Kováč, 2009).

Although black bullhead are omnipresent in many aquatic habitats within their native range, there is still limited research on the biology and life history traits of this species (Scott & Crossmain 1973; Brown et al., 1999; Novomeská & Kováč, 2009; Copp et al., 2016). Much of the focus of the research on black bullhead explores its invasiveness in many European countries (Wheeler, 1978; Cucherousset et al 2008; Kreutzenberger et al., 2008; Rutkayová et al., 2008; Copp et al., 2016) where it directly affects native species through the reduction of native prey available for native predators, generates turbidity which affects the foraging and feeding efficiency of visual predators, and its overall abundance which interferes with the natural biological processes of native species (Kreutzenberger et al., 2008; Coppet al., 2016). The success of the black bullhead as an invader can be attributed to its ability to tolerate extreme environmental conditions (i.e., low dissolved oxygen, warmer water temperatures), its ability to alter its reproductive responses resulting in earlier maturation, and multiple spawning events which lead to an overall high abundance of this species in non-native habitats (Novomeská et al., 2009).

Black bullhead have been used as the study subject to investigate its responses to various types of stimuli. Black bullhead have been exposed to anthropogenic boat noise to determine its effects on the behaviour and physiology of this fish (Mickle et al., 2018; Pieniazek et al., 2020). Mickle et al. (2018) determined that boat noise exposure (140 dB re 1 μPa) caused a decrease in activity levels and an increase in sheltering during noise trials, as well as a decrease in ciliary bundles at higher noise intensities (160 and 170 dB re 1 μPa) suggesting that higher noise intensities can affect both the physiology and behaviour of this fish. Pieniazek et al. (2020) specifically tested the effects of anthropogenic boat noise on foraging in black bullhead as well as other freshwater fish species with varying hearing capabilities. Both laboratory and field experiments were conducted and revealed that boat noise decreases foraging behaviour and the presence of wild fishes within the area of the sound source. Their results showed that the effects of noise were species-specific meaning that the species with enhanced hearing (species containing the Weberian apparatus) were more affected in terms of foraging behaviour than other study species with average hearing abilities. Perhaps black bullhead may be prone to more inner ear damage due to their enhanced hearing abilities and therefore may be more affected by a wide range of sounds. The olfactory studies done on black bullhead have determined how they can discriminate between amino acids that play an important role for hunting and localizing food sources and effects of pollutants and chemicals that affect the morphology of their olfactory epithelia (Zeni & Stagni, 2002; Valentinic et al., 2011; Dolensek & Valentincic, 2010). There is great potential and opportunities for new research avenues to be explored with the black bullhead. Black bullheads make a good study subject for a wide variety of experiments, specifically ones that involve assessment of hearing abilities due to their enhanced hearing. Continuing research in these fields can aid in filling the knowledge gaps of the biology of an abundant freshwater fish species.

Thesis Objectives

Through experimental analysis, my thesis aims to address how various stimuli can affect the behaviour and physiology of the round goby (*Neogobius melanostomus)* and the black bullhead (*Ameiurus melas*), respectively. The first objective of chapter 2 was to investigate whether reproductive male round gobies can exhibit synergistic responses to multimodal stimuli. This was accomplished by presenting males with both unimodal stimuli and multimodal stimuli through behavioural experiments. I presented fish with acoustic, visual, and olfactory stimuli both individually and simultaneously to elicit a behavioural response. The time spent resting, approaching stimuli, swimming, erratic swimming, and average respiration rate were the behaviours that were quantified during experiments. I hypothesized that male round gobies will respond to both unimodal and multimodal stimuli and will exhibit a synergistic response to multimodal signals. I predicted that the time spent swimming, erratic swimming, and approaching stimuli will increase in response to multimodal stimuli and will be greater than the responses to unimodal stimuli. Additionally, I hypothesized that the time spent resting will decrease as the experiment progresses and that the average respiration rate will show an increase when presented with multimodal stimuli. The second objective of chapter 2 was to determine if the reproductive maturity of the round gobies used in experiments influence the behavioural responses. I predict that the subjects with higher reproductive maturity will show behavioural responses that are more intense and therefore resulting in a synergistic response.

Chapter 3 focuses on the impact of noise on black bullhead. This was investigated by conducting two separate studies which aimed to assess the impacts of anthropogenic boat noise exposure and regenerative capabilities of ciliary bundles in black bullhead. The first objective of Experiment 1 was to determine if black bullhead can regenerate ciliary bundles of the inner ear following 1.5 hours of boat noise played at 170 dB re 1 μPa. The second objective of this experiment was to determine the time course of this regeneration. I hypothesized that the fish would exhibit moderate damage and decrease of ciliary bundle counts but will be able to regenerate lost ciliary bundles to control levels. The main objective of Experiment 2 was to determine how regeneration varies with increasing sound intensity when exposed to 2 hours of anthropogenic boat noise played at 160, 165, 170, and 175 dB re 1 µPa. I hypothesized that the fish would exhibit moderate damage to ciliary bundles when exposed to boat noise played at 160, 165 dB re 1 µPa and the most damage will be seen when exposed to 170 and 175 dB re 1 µPa. In all instances, I predict that ciliary bundle counts will regenerate to control levels and that higher sound intensities will require a longer recovery period to regenerate ciliary bundles to control levels. With this research, I hope to bring and understanding to the behavioural responses of the round goby in a laboratory experiment that integrate multiple signals to emulate multimodal communication in fishes. I also intend to highlight the impacts of noise and regeneration in the black bullhead to bring awareness of the negative impact anthropogenic noise continues to have in freshwater habitats and its native species.

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CHAPTER 2

INTEGRATIVE RESPONSE OF THE ROUND GOBY (*NEOGOBIUS MELANOSTOMUS*) TO MULTIPLE STIMULI

Introduction

Fish are surrounded by multiple stimuli within their habitats which provides important information about the complexity of their environment (Rowland, 1999; McCormick & Manassa, 2008; Touhara, 2013) allowing them to make important decisions and respond appropriately to surrounding stimuli, benefiting their survival (Rowland, 1999). Sensory signals are evolved stimuli that are used in the context of communication and can encompass a range of sensory modalities (Marshall, 2011). Fish communication can be in the form of visual, acoustic, and olfactory/chemical signalling as well as electrocommunication and mechanosensation. Previous research has used a variety of stimuli on fish to elicit behavioral responses with the aim of understanding how animals behave in their natural environments but most of this previous work has focused on one sensory stimulus at a time, even though fish often use a multitude of sensory systems simultaneously to process environmental information (Partan, 2013).

Many fish species rely on sound as a form of communication during agonistic interactions, aggressive encounters, during courtship and spawning behaviours, when frightened or feeding (Amorim, 2006; Kasumyan, 2009; Fine & Parmentier, 2015). Depending on the species, fish can produce vocalizations that range from low to high frequencies, with high frequency calls linked to species with enhanced hearing (Ladich, 1997). Vocalizations may vary through repetition rate and duration (Ladich, 1997; Amorim, 2006). There is great diversity in the mechanisms fish use to produce sounds with the two most common being bone stridulation and the sonic swimbladder system (Amorim, 2006; Kasumyan, 2008; Fine & Parmentier, 2015). Acoustic playback experiments use a variety of sounds as acoustic stimuli to investigate how fishes respond to acoustic cues in various contexts. Playback experiments can explore how vocal fishes produce and localize calls during courtship and spawning events, which provides insight on how fishes interpret vocalizations and how it may influence mate assessment (Malavasi et al., 2003; Amorim, 2006; Rollo & Higgs, 2008; Kasumyan, 2009; Amorim et al., 2013 Isabella-Valenzi & Higgs, 2013). The reproductive males of many vocal fish species exhibit unique courtship behaviours and sounds to attract females during breeding season (Kasumyan, 2009). Among the well-studied vocal fish species are the plainfin midshipman (*Porichthys notatus*) and Lusitanian toadfish (*Halobatrachus Didactylus*), which belong to the Batrachoididae family, where they have been used a model to understand in sound production in terms of the reproduction, behavioural and neurophysiological aspects of sound (Amorim, 2006; Sisneros, 2009a; Sisneros, 2009b). Vocalizing fish are not limited to the Batrachoididate family as there exists a multitude of vocalizing fish families including species belonging to the Gobiidae, Sciaenidae, Percidae, and Acipenseridae that are known to make sounds (Amorim, 2006; Kasumyan, 2009). Anthropogenic noises may also be used as acoustic stimuli to understand the impact anthropogenic noise (i.e., shipping noise, pile driving, dredging etc.) has on fish in terms of types of stressors it may cause which ultimately affects their survival (Mickle & Higgs, 2018.)

Vision and the use of visual cues are also important in many fish species. Visual displays are common for agonistic and courtship contexts, as they can communicate a wealth of information when fish are in close proximity (Kelley et al., 2012; Neri, 2020). Visual experiments often use mirrors, live fish, dummies, or video playback as visual stimuli to elicit and/or modify behavioural responses in fish (Lattal & Metzger, 1994; Rowland, 1999; Gonçalves et al., 2000; Dzieweczynski & Leopard, 2010) which helps researchers understand courtship rituals, aggressive behaviour, and sexual selection in fish (Rowland, 1999). Studies of this nature can also investigate the effects of habituation, priming, where pre-exposure to a stimulus may influence certain behaviours, or visual lateralization (Peeke & Peeke, 1970; Sovrano, 2004; Matos et al., 2003).

Olfactory signalling plays an important role in fish reproduction, species recognition, migration shoaling and predator avoidance (McCormick & Manassa, 2008; Touhara, 2013). Pheromones are commonly defined as odours or odour mixtures that are released by a sender, evoking a species-specific response by the receiver, and are comprised of soluble steroids, bile acids or proteins (Burnard et al., 2008). Pheromones may induce releaser responses which cause rapid changes to behaviours or primer responses which cause slower physiological effects (Burnard et al., 2008). Pheromones are particularly effective in environments with low visibility allowing them to travel far distances regardless of light levels. Fish species like the common goldfish (*Carassius auratus*) or the Atlantic salmon (*Salmo salar*) use pheromones to attract potential mates for spawning and invasive freshwater species such as the round goby *(Neogobius melanostomus)* and the sea lamprey (*Petromyzon marinus*) also rely on the release of pheromones during reproduction (Corkum et al., 2006; Burnard et al., 2008). Using pheromones as a trapping method may aid in controlling invasive populations to reduce the competition for native species and allow them to thrive. The use of pheromones as olfactory stimuli in experiments can provide insight on the specific behavioural responses that can be elicited from olfactory cues. Olfactory studies also investigate pheromone detection in other contexts like predation risk assessment or alarm cues, which are involuntarily triggered when skin damage occurs, and chemical cues are released to indicate that there is a predator in the area (McCormick & Manassa, 2008). There is still some debate whether alarm substances can be considered true pheromones but nonetheless still play a crucial role in predator avoidance.

Behavioural studies involving the presentation of a stimulus have mainly focused on single (Mclennan & Ryan, 1997; Atema et al., 2002; Smith et al., 2002) or coupled sensory modalities (Plenderleith et al., 2005; Bertucci et al., 2010; Kasurak et al., 2012; Maruska et al., 2012) but it is important to continue to test the effects of integrating multiple stimuli on fish and how their synergistic responses can aid in reducing the time it takes for the receiver of a signal to interpret and respond to more than one stimulus (Barry et al., 2010; Kasurak et al., 2012). A synergistic response to multimodal signalling can be defined as the intensity of a behavioural response being greater than or different from the sum of individual responses to unimodal signals. (Wisenden et al., 2003; Partan & Marler, 2005; Kasurak et al., 2012). There is limited research on multimodal signalling in fish but there are some studies that investigate multimodal signalling among a variety of taxa (Acquistapace et al., 2002; Moller, 2002; Wisenden et al., 2003; Mikheev et al., 2006; Uetz et al., 2009). Although many studies have focused on unimodal signalling there is a rise in exploring multimodal signalling and how a variety of taxa including fish can respond to multimodal signals.

In the current study, I used the round goby (*Neogobius melanostomus*) to test the role of multiple sensory stimuli in behavioural responses. The round goby is a benthic fish that belongs to the Gobiidae family of vocalizing fish (Lingström & Lugli, 2000) and is native to the Black and Caspian seas. It has been introduced to the Great Lakes by the ballast water of foreign vessels originating in the Ponto-Caspian region (Corkum et al., 2004; Kornis et al., 2012). The round goby has successfully invaded many ecosystems due to its ability to adapt to a wide range of environments, its broad diet, its ability to spawn repeatedly throughout the spring and summer and its aggressive behaviour (Charlebois et al., 2001; Corkum et al., 2004). Known aggressive goby behaviours include biting, approaching, raising pectoral and dorsal fins, and emitting calls (Groen et al., 2012). They use these aggressive behaviours to defend their nest as well as to displace native species from their habitats (Bergstrom & Mensinger, 2009). The reproductive strategy of the round goby involves emitting courtship calls consisting of slow pulses or pulse trains, faster tonal sounds and complex sounds that combine the two (Lugli et al., 1997; Amorim & Neves, 2007) as well as the release of pheromones (Corkum et al., 2006) to attract mates to the nest and induce spawning (Andraso et al., 2007).

The objective of this chapter was to present acoustic, visual, and olfactory stimuli to reproductive male round gobies, separately then simultaneously, to test for a synergistic or enhanced response. The time spent swimming, erratic swimming, approaching stimuli, resting and the average respiration rate were the quantified behaviours in this study. I hypothesized that male round gobies would exhibit a synergistic response to multimodal signalling (acoustic, visual, and olfactory) and that their behavioural responses to unimodal signalling would be weaker and less intense than the response to multimodal signalling. I hypothesized that presentation of multimodal stimuli will cause an increase in time spent swimming, erratic swimming, and approaching stimuli resulting in a greater and more intense response than responses to unimodal stimuli. I also predicted that respiration rate will increase in response to multimodal stimuli and that the time spent resting will decrease as the experiment progresses. Finally, I aimed to determine if reproductive status influences the behavioural responses to stimuli and that higher reproductive maturity can result in an enhanced behavioural response compared to individuals that have low reproductive maturity.

Methods

General Methods and Experimental Set-Up

All experimental fish (n=20) ranged in size from 7.9-11.5cm in total length (TL) and were caught at two locations: 1) Mckee park (n=15) in Windsor Ontario (42°18'23.7"N 83°04'30.7"W) and 2) Lasalle, Ontario, Canada at two locations within Riverdance Park (n=5) (42°14'09.6"N 83°06'19.6"W and 42°14'14.1"N 83°06'25.3"W). All except one fish were caught by angling. One fish was caught by seine fishing. Fish were transported and housed at the University of Windsor Central Animal Care Facility (September 2020-December 2020) and maintained following Canadian Council on Animal Care (CCAC) guidelines with the following conditions: temperature of 14-16 ˚C, pH of 6.5-7.5 and a 12:12 light/dark cycle. Experiments took place in a soundproof room where an 18 L glass tank (16” x 8” x 8.5”) was equipped with an underwater speaker (Electro-Voice UW-30), placed behind a mesh barrier to prevent fish from sheltering underneath, and was connected to an amplifier (Pioneer Max Power 400W) powered by a rechargeable battery (Leoch 12 V). A GoPro Hero 7 was used to record trials and an iPad Mini 3 (Apple Inc) was placed against the exterior of the tank to play visual and acoustic stimuli. A red light was used to illuminate the tank during trials. The olfactory stimulus was introduced via an apparatus that consisted of a 5.6 L container (4.59”x 8.19”x 13.4”) with a hole at its base connecting an ¼” IV tube (Nalgene 180 PVC non-toxic autoclavable Lab/FDA/USP VI Grade – ¼” ID) with a lever to control the flow of conditioned water into the experimental tank. The apparatus was mounted outside of the soundproof room and administered 1 L of pheromones for each olfactory stimulus, which took 3 minutes to empty into the tank.

Experiments required the use of both reproductive male and female round gobies. Males were used during experiments and were exposed to acoustic, visual, and olfactory stimuli and females were used to collect conditioned goby water which was used as the olfactory stimulus for trials. Reproductive males were primarily identified by the enlarged urogenital papilla (narrow and pointed) on the ventral side of the body near the anus (Marentette et al., 2009) but were also assessed for swollen cheeks and black colouration (Marentette et al., 2009). Reproductive females were chosen by assessing the urogenital papilla located on the ventral side of the body near the anus, which becomes swollen and orange when in colour when they are reproductive (Marentette et al., 2009).

Experimental Stimuli

The acoustic stimulus used in experiments was a recording of a male round goby pulse call which was amplified by 15 dB and played at 150 dB re 1 µPa. This sound intensity falls withing the hearing range of the round goby (100-600hz at 145-160 dB re 1 µPa; Belanger et al., 2010) therefore, there is no concern for potential hair cell damage or hearing loss when presented with calls at this intensity. The visual stimulus used was a recording of male reproductive round goby exhibiting resting behaviour for 2.5 minutes followed by swimming behaviour for 2.5 minutes. 1L of conditioned goby water was used as the olfactory stimulus and was created by placing a reproductive female round goby in

1L of dechlorinated water for 4 hours (Gammon et al., 2005). The conditioned water was either used the same day for experiments or stored in the freezer for later use. Frozen conditioned water was used within 1-2 days of collection.

Experimental Trials

Experiments were 1 hour and 15 minutes in length. A 30-minute acclimation time was determined in the pilot trials to be sufficient time for the fish to acclimate to its new surroundings. The control and stimuli periods were each 5 minutes in length. The following sequence of control and treatment was presented to each fish for each trial: acoustic control, acoustic stimulus, visual control, visual stimulus, olfactory control, olfactory control, control for all stimuli and ending with the presentation of all stimuli simultaneously (Fig. 2.1). The controls for each stimulus consisted of a dark screen, no sound, and no introduction of pheromones. At the end of each trial, the fish was observed for an additional 5 minutes (post-experiment period). Following the post-experiment period, each fish was euthanized using 2-phenoxyethanol and body measurements were taken which included: body weight (g), total length (cm) and head width (cm) and preserved in 95% ethanol. An incision on the ventral side of the fish was made so that the inner organs and gonads would be preserved for further dissection of gonads to calculate the gonadosomatic index (GSI). The gonads were removed from the fish and weighed (g) and the gonadosomatic index formula was used to determine reproductive condition of each fish.

$$(GSI = \frac{(gonad\ weight\ (g))}{(body\ weight\ (g))} \times 100\%)$$

Video Analysis

Video analysis evaluated the total time spent exhibiting the following behaviours: swimming, erratic swimming, approaching stimuli, and resting. Swimming behaviour was defined as swimming less than a body length towards a stimulus, swimming more than a body length that was not unidirectional and turns. Erratic swimming was defined by swimming vertically up and down the water column haphazardly. An approach was defined by the fish traveling at least one body length in a straight line towards a physical stimulus (speaker or iPad). The average respiration rate was also quantified by evaluating opercular movements for every minute that the goby was at rest. These behaviours were also quantified in the post-experiment period for further analysis.

Statistical Analysis

Using the statistical analysis program SPSS (version 28, IBM SPSS Statistics, Chicago, IL) a repeated measures analysis of variance (ANOVA) was performed for the current study for each behaviour. The independent variables were the acoustic, visual, and olfactory treatments that were presented to each fish and the dependent variables were the quantifiable behaviours observed in each experiment. Each subject within the study was exposed to 4 treatments and repeated observations were taken on each test subject therefore, it was deemed appropriate to run the repeated measures test on the data. Prior to running the repeated measures ANOVA on the data set, separate tables were created for each behaviour where time spent exhibiting behaviours (in seconds) were recorded. Experimental values were subtracted from the control values and then a repeated measures ANOVA was run on the differences between control and experimental values as a simple contrast model compared to theoretical distribution of zero so that each fish could serve as its own control (Field, 2013). A significant main effect then signifies that the experimental treatment caused a change from control conditions, analogous to the procedure used in a one-sample t-test but appropriate for the repeated measures ANOVA analysis used here (Field, 2013). When a significant main effect was identified, post-hoc comparisons were tested to determine which treatment differences significantly deviated from zero. A sequential Bonferroni test was conducted to control for the type I error and correct for multiple comparisons since multiple observations were being quantified per subject. Significant results were ranked from smallest to largest (more significant to less significant) where each behaviour was compared to its own significance level which became more stringent as significant *p*-values increased. Five distinct significance levels were used for each quantified behaviour (average respiration rate, $\alpha = 0.05$; approach, $\alpha = 0.025$; rest, $\alpha = 0.0166$; swim, $\alpha = 0.0125$; erratic swim, $\alpha = 0.001$). Pair-wise comparisons were made where differences occurred. Additionally, a repeated measures ANOVA was conducted with fish size and source location as covariates in SPSS to determine their effects on behavioural responses. Maulchy's test of sphericity was used for the repeated measures ANOVA. If the data violated Maulchy's test of sphericity then the Greenhouse-Geisser was used as a correction (Field, 2013) (Table 2.1). Five additional olfactory control trials were conducted to compare the use of dechlorinated water as an olfactory control instead of a period of no conditioned water as a control. It was determined there was no significant differences between either control method ($p>0.05$) for all cases.

Results

There was a significant main effect of treatment on average respiration rate ($F_{2.937,55.800}$=6.859, *p*<0.001), with post-hoc comparisons showing the olfactory stimulus (*p*=0.001) and the combined AVO (acoustic, visual, and olfactory) stimulus (*p*=0.009) caused a significant increase in respiration rate (Fig. 2.2). A decreasing trend in respiration rate was seen when presented with the acoustic and visual stimuli (*p*>0.05) which were not significant.

There was no significant main effect of treatment on time spent approaching after sequential Bonferroni corrections ($F_{1.436,27.283}$=4.410, *p*=0.033). The graphical data showed increasing trends in time spent approaching stimuli during the olfactory and combined AVO stimulus but was not significant in this case (*p*>0.025) (Fig. 2.3). There was no significant effect of treatment on time spent resting ($F_{2.421,43.572}$=3.080, *p*=0.047). The graphical data showed decreasing trends of the time spent resting during the combined AVO stimulus but was not significant in this case (*p*>0.0166) (Fig. 2.4). There was no significant main effect of treatment on time spent swimming ($F_{2.155,40.179}$=1.874, *p*=0.165). The graphical data showed decreasing trend of the time spent swimming during the visual stimulus and the combined AVO stimulus but were not significant for those cases (*p*>0.0125) (Fig. 2.5). Finally, there was no significant main effect of treatment on time spent erratically swimming ($F_{1.870,35.530}$=0.962, *p*=0.387). The graphical data also shows increasing trends during the visual stimulus and the combined AVO stimulus but were not significant for those cases (*p*>0.001) (Fig. 2.6).

The gonadosomatic index (GSI) was calculated for each fish revealing that fish #11 (GSI= 4.145%), #12 (2.919%), #14 (3.462%) and #19 (3.726%) were reproductive males while all other fish were non-reproductive males (GSI<1%) (Fig. 2.7). The behavioural responses of the 4 fish mentioned above did not drastically differ from all non-reproductive males used in experiments. The data for the time spent exhibiting each behaviour of reproductive males were graphically compared to the times of non-reproductive males and showed that the behaviours of the reproductive males fell within the distribution of the rest of the data; this trend was also seen for all behavioural responses (Fig. 2.8 A-E). Statistical analysis revealed that there were no significant location effect and effect of size on all behavioural responses, so the model was collapsed without these effects for all cases ($p>0.05$ for all cases) (Table 2.2 & Table 2.3)

Discussion

The main objective of this chapter was to determine if reproductive male round gobies can exhibit a synergistic response to multimodal signals and if reproductive status can influence the type of behavioural responses to multimodal signals. The male round gobies used in this study were responsive to the unimodal and multimodal signals but showed more affinity to responding to multiple stimuli. My first hypothesis of males exhibiting a synergistic or enhanced response to multimodal signalling was supported by the data. The data suggests that the fish may be responding synergistically to multimodal stimuli through the decrease in the average respiration rate during the olfactory stimulus and the combined AVO stimulus. The time spent approaching, resting, swimming and erratic swimming were not significant but showed increasing trends in the graphical data during the combined AVO stimulus, that may be elucidated with a larger sample size.

The average respiration rate decreased when presented with the olfactory stimulus and the combined AVO stimulus, with the greatest decrease seen during the olfactory stimulus. Previous research has shown that reproductive male round gobies exhibit an increase in gill ventilation when exposed to the putative pheromone, estrone (estrone, (1,3,5(10)-estratrien-3-ol-17-one) and gonadal extracts (Belanger et al., 2006; Belanger et al., 2007) of reproductive female round gobies. It would be intuitive to expect this behavioural response to the olfactory stimulus used for experiments, however Belanger et al. (2007) also reported that reproductive male round gobies were more sensitive to smaller concentrations (10^{-8} – 10^{-11} M of Estrone) of putative pheromones than their non-reproductive counterparts (10^{-8} – 10^{-9} M of Estrone) with a 100-fold molar difference in sensitivity to this odorant and responded by increased gill ventilation. In the current study, sixteen out of twenty fish that were used were non-reproductive males resulting in only four reproductive males. This could be why the fish did not exhibit an increase in respiration rate concluding that reproductive state can influence the behavioural responses to the olfactory stimulus used here. Even though an increase in respiration rate was not observed when presented with the stimuli and there was no significant effect on the time spent approaching, resting, swimming and erratic swimming, it does not indicate that no information is being communicated (Marentette & Corkum, 2008). Non-reproductive males may not respond at all or show the same types of responses as their reproductive counterparts since the signals might not relevant and worth the energetic expense at that particular life stage. The reproductive individuals used in this study were small Type II sneaker males that did not exhibit secondary sexual characteristics (puffy cheeks or black colouration) but had an enlarged urogenital papillae and large testes. The behavioural responses of reproductive males did not differ greatly from non-reproductive males, nor did they show any preference for a particular behaviour. To further explore differences between behavioural responses of different morphs, comparisons of Type I and Type II males should be investigated to how response may vary with reproductive status as well as individuals that also show secondary sexual characteristics.

Future studies should increase the sample size to elucidate the trends seen in the graphical data for approaching, resting, swimming, and erratic swim behaviours. The current data shows that males may be spending more time approaching stimuli when presented with the combined AVO stimulus than with unimodal stimuli. It is possible that the olfactory stimulus may be driving the approaching behaviour during experiments since the presentation of the olfactory stimulus precedes the combined AVO stimulus. Unlike the acoustic and visual stimulus, it may be difficult for the fish to localize the source of the olfactory stimulus since the apparatus used to administer the pheromones is not easily seen. The underwater speaker and the iPad used in experiments are placed on opposite sides of the tank and are physical structures than can aid in localizing the source of the acoustic and visual stimuli. Searching for the source of the olfactory stimulus may induce a searching behaviour and increase approaching behaviours during the combined AVO stimulus. In this instance, the olfactory stimulus may be acting as a releaser which causes immediate effects on behaviour influencing the fish to approach more during multimodal signalling which can be seen in studies involving behavioural responses to putative pheromones (Li et al., 2003; Guevara-Fiore et al., 2010). Studies that expose the round goby to putative pheromones have shown immediate behavioural responses to these stimuli such as increased gill ventilation (Murphy et al., 2001; Belanger et al., 2006), increase time spent in the stimulus zone (Corkum et al., 2008; Kasurak et al., 2012) or overall attraction to released olfactory stimuli (Belanger et al., 2004; Gammon, 2005). The experiments aimed to explore not only synergism in the behavioural responses but also integration of behaviours in response to multimodal signals as well. While the sample size of this study may limit the ability to make firm conclusions regarding integration of behaviours, the data suggests that all physical behavioural activity (i.e., approaching, swimming, erratic swimming) may be linked to the average respiration rate. As more activity occurs as the experiment progresses, there is less time that is spent resting and a decrease in average respiration rate is observed, which in this case could mean that it is more beneficial to respond to signals through movement. As fish spend more time within the tank during experiments, they may be becoming more acclimated to their environment resulting in more exploration of their surroundings and a decrease in respiration rate. As previously mentioned, the non-reproductive status of the fish may be influencing their decreased respiration rate in response to the olfactory stimulus as it may be not a relevant signal to respond to.

The time spent resting showed decreasing trends when exposed to unimodal stimuli but showed the greatest decrease in resting behaviour when exposed to the combined AVO stimulus (acoustic, visual, and olfactory). Further testing could reveal that multimodal signalling may elicit a greater behavioural response than unimodal signals and that multimodal signals can drive more behavioural activity. Swimming and erratic swimming behaviours showed similar trends with increased time spent exhibiting these behaviours during the visual stimulus and the combined AVO stimulus. Perhaps the visual stimulus may be the factor that is driving these behaviours. The trends in the data may be further clarified by increasing the sample size of the study.

Although the main goal of the current study was to investigate synergistic responses in round gobies to multimodal stimuli, it is important to make distinction between additive responses and synergistic responses. A synergistic response to multimodal stimuli results in a response that is greater than the sum of responses to individual stimuli (Wisenden et al., 2003; Partan & Marler 2004; Kasurak et al., 2012). In contrast, an additive response to multimodal stimuli considers the individual responses to unimodal stimuli and sums them together to obtain a final measure of the response in question (Moreno-Marin et al., 2018; Braga et al., 2020). If the behavioural responses to multimodal stimuli are greater than the sum of responses to individual stimuli, then the response can be categorized as synergistic. When adding the individual response of unimodal stimuli and comparing those values to the behavioural responses to the combined AVO stimulus, the data shows that the average respiration rate is greater than the sum of individual components. Therefore, a synergic response to multimodal stimuli may be occurring during experiments through a decrease in respiration rate in response to the combined AVO stimulus.

Studies presenting multimodal stimuli and specifically three types of stimuli to round gobies are limited. Both non-reproductive and reproductive female round gobies were presented with reproductive male calls and male conditioned water, separately and simultaneously in a study conducted by Kasurak et al. (2012). They determined that only female reproductive round gobies exhibited a synergistic response to multimodal signals and that reproductive status plays a role in attraction and localizations of reproductive signals. Synergistic responses to multimodal signalling have also been investigated in other taxa where multimodal stimuli are more attractive and more often induces an enhanced behavioural response and benefit prey detection schooling or shoaling, foraging and courtship (Acquistapace et al., 2002; Moller, 2002; Wisenden et al., 2003; Mikheev et al., 2006; Uetz et al., 2009).

Using multimodal signalling can increase the accuracy of a signal and ensure that the intended message communicated and responded to appropriately (Partan & Marler, 2005; Kasurak et al., 2012). Providing more than one signals simultaneously can decrease the risk of signal degradation over long distances (Kasurak et al., 2012). Previous studies have used only two types of stimuli while my study uses three; testing with more stimuli can provide a better understanding of how fish respond to multiple stimuli which accurately represents their natural environment. Two studies have used round goby and their response to multiple stimuli (Yavno & Corkum, 2010; Kasurak et al., 2012) limiting the research in this field. My study can shed light on integrative responses in freshwater fish in a laboratory setting and can be the steppingstone to determine if multimodal signalling is worth the energetic expense and if its use is a good indicator of fitness.

Currently there are no studies that directly compare behavioural responses of invasive populations to native populations (Ponto-Caspian region: Black and Caspian Seas) of the round goby. Studies have explored the ecological impact of round gobies on invaded habitats throughout Europe and the Laurentian Great lakes but there have not been direct comparisons in behavioural responses to different types of sensory modalities. Previous research has compared the DNA sequences of the native populations of round gobies to that of the non-native populations in the freshwaters of Europe and the Laurentian Great Lakes. (Stepien et al., 2005; Stepien & Tumeo, 2006; Brown & Stepien., 2008; Gutowsky & Fox, 2012) and variations in external morphology in invasive populations (Dashinov et al., 2020) have also been determined. Genetic analysis has shed light on the genetic makeup of populations of round gobies in the Great Lakes and has revealed that there was high genetic diversity among the individuals of round goby populations in the Great Lakes and that there were little to no founder effects (Stepien et al., 2005; Stepien & Tumeo, 2006). Founder effects occurs when there is a reduction in genetic variability due to the establishment of a small subset individuals of a large population with low genetic diversity, resulting in genotypes and phenotypes in the new colony that may differ from the native population. It is widely accepted that there were multiple introductions of the round goby to the Great Lakes which resulted in highly genetically diverse individuals adding to their ability to thrive and adapt to new environments adding to their success as invaders (Stepien & Tumeo, 2006; Brown & Stepien, 2008; Synder & Stepien, 2017). Interestingly, there seems to be diversity within sympatric populations showing that this species is highly adaptable even within one region (Stepien & Tumeo, 2006; Bronnenhuber et al., 2011; Gutowsky & Fox, 2012). Even though the non-native populations have conserved the high genetic diversity of their native counterparts, they may vary in behavioural responses when exposed to the same type of stimuli. Since both populations are in different environments that have different pressures and stressors each may exhibit varying responses relative to their environment. Perhaps behavioural responses to multimodal stimuli within their environment may vary among native and non-native populations of this species and may be more hypersensitive to different signals and can maximize their life history traits to survive in new environments, but much more research is needed in this area for this to be determined. With the current study and previous research (Kasurak et al., 2012) showing that round gobies respond synergistically to multimodal stimuli, developing multimodal traps to capture and remove round gobies from their non-native habitats may be a solution towards the management of this species. Previous research has suggested using acoustic traps as a method to manage this species (Isabella-Valenzi & Higgs, 2016) but incorporating multiple relevant signals in a multimodal trap can make the management of this species more efficient. Future studies should consider conducting experiments in the field to further develop multimodal traps so that they function appropriately and effectively within the non-native habitats of round gobies.

There are some limitations of the current study. A larger sample size would increase the strength of the study as well as randomization of the presentation of stimuli to investigate priming effects on behaviour which may alter behaviours as the experiments progress. The order of the stimuli presentation was not randomized to avoid priming by the olfactory stimulus. Presenting the olfactory stimulus at the end of the trial would ensure that the tank would not have any residual pheromones within the tank that could influence behaviour. It would be difficult to rid the environment of pheromones without interrupting the progress of the experiment. A larger sample size could elucidate trends seen in the data for behaviours that showed increases but were not significant. With a larger sample size, we may delve deeper into understanding the full effect the stimuli had on other behaviours. The four non-significant behaviours were marginally significant prior to the multiple comparison's tests conducted (Sequential Bonferroni), so perhaps a larger sample size could use a different statistical test that would be appropriate to the sample size. In terms of the experimental setup, substrate and shelter can be added to the tank to simulate a natural environment to observe how the fish usually interact within their habitats. Coupled with choosing more reproductive fish and a simulated natural environment, we may be able to observe reproductive tactics in the laboratory setting.

Future studies can directly compare behavioural responses of both non-reproductive morphs to reproductive morphs of males and female round gobies and Type

I males (nest-guarding males) to Type II males (sneaker males) of this species to observe how behavioural responses differ with reproductive status. Previous research has shown that reproductive maturity can influence behavioural responses and drive certain behaviours in many fish species, we can expect different responses in reproductive round gobies. This field can also continue to use multiple sensory modalities, specifically three or more sensory modalities to explore synergistic responses to multimodal signalling and how reproductive status not only effects behavioural responses but the sensory systems as a whole. Not only can it elucidate the biological processes occurring during the production and perception of multimodal signals in the round goby but can also reveal how multimodal signalling may be one of the factors that drives its success as an invader potentially leading to strategies to prevent further spread to vulnerable habitats.

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Behaviour	**Maulchy's W**	**df**	**Significance**	**Greenhouse-Geisser**
Respiration Rate	0.160	9	<0.001	0.734*
Approach	0.001	9	<0.001	0.359
Rest	0.047	9	<0.001	0.605
Swim	0.037	9	<0.001	0.529
Erratic Swim	0.009	9	<0.001	0.468

Table 2.1 Greenhouse-Geisser corrections

*Where Greenhouse-Geisser corrections were needed, F-values in text are after Greenhouse-Geisser correction

Behaviour	**df**	**Error**	**F-value**	***p*-value**
Respiration Rate	2.537	22.8311	0.775	0.661
Approach	1.004	8.029	0.528	0.489
Rest	1.565	12.523	0.608	0.521
Swim	1.235	9.883	0.273	0.992
Erratic Swim	1.763	14.108	0.871	0.427

Table 2.2 Location Effects

A repeated measures ANOVA was conducted with location (Lasalle or Chewitt) as a covariate in SPSS to determine location effects on the round gobies used in the study. Statistical analysis revealed that there was no significant location effect on behavioural responses, so the model was collapsed without location effect in all cases.

Behaviour	**df**	**Error**	**F-Value**	***p*-value**
Respiration Rate	2.870	51.667	1.031	0.384
Approach	1.432	25.773	0.195	0.750
Rest	2.403	43.257	0.097	0.936
Swim	2.109	37.965	0.096	0.918
Erratic Swim	1.837	33.066	0.428	0.669

Table 2.3 Effect of Size of Fish on Behavioural Responses
A repeated measures ANOVA was conducted with fish size as a covariate in SPSS to determine size effects on the round gobies used in the study. Statistical analysis revealed that there was no significant of size on behavioural responses, so the model was collapsed without location effect in all cases.

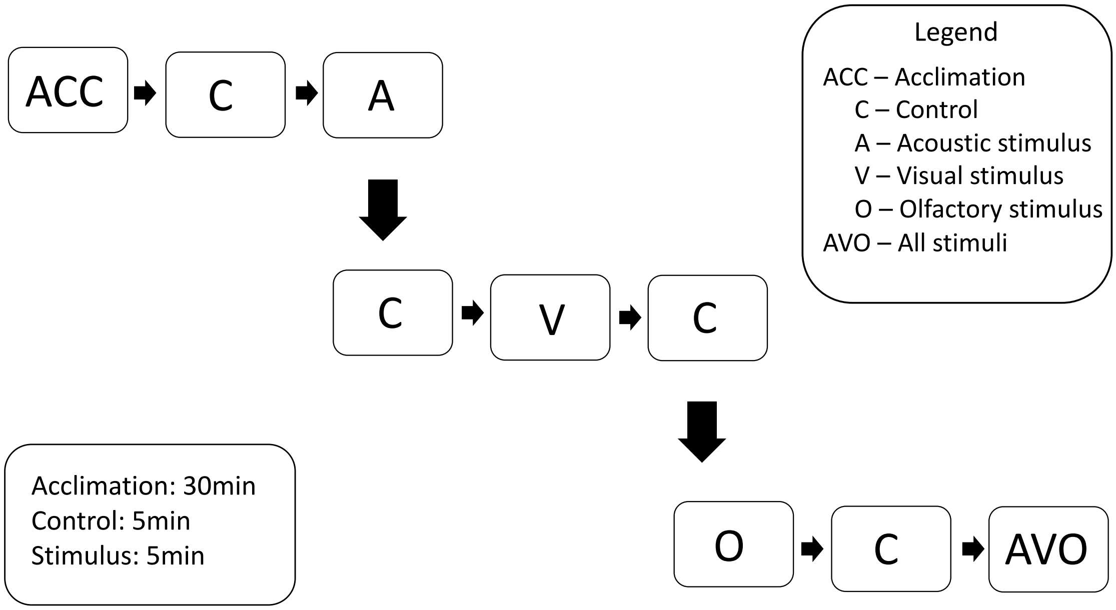


Figure 2.1 Experimental Sequence

Each experiment followed this specific sequence of presentation of control and stimuli. Each experiment began with a 30-minute acclimation time followed by alternating between control and the chosen stimulus ending the experiment with presentation of all stimuli simultaneously.

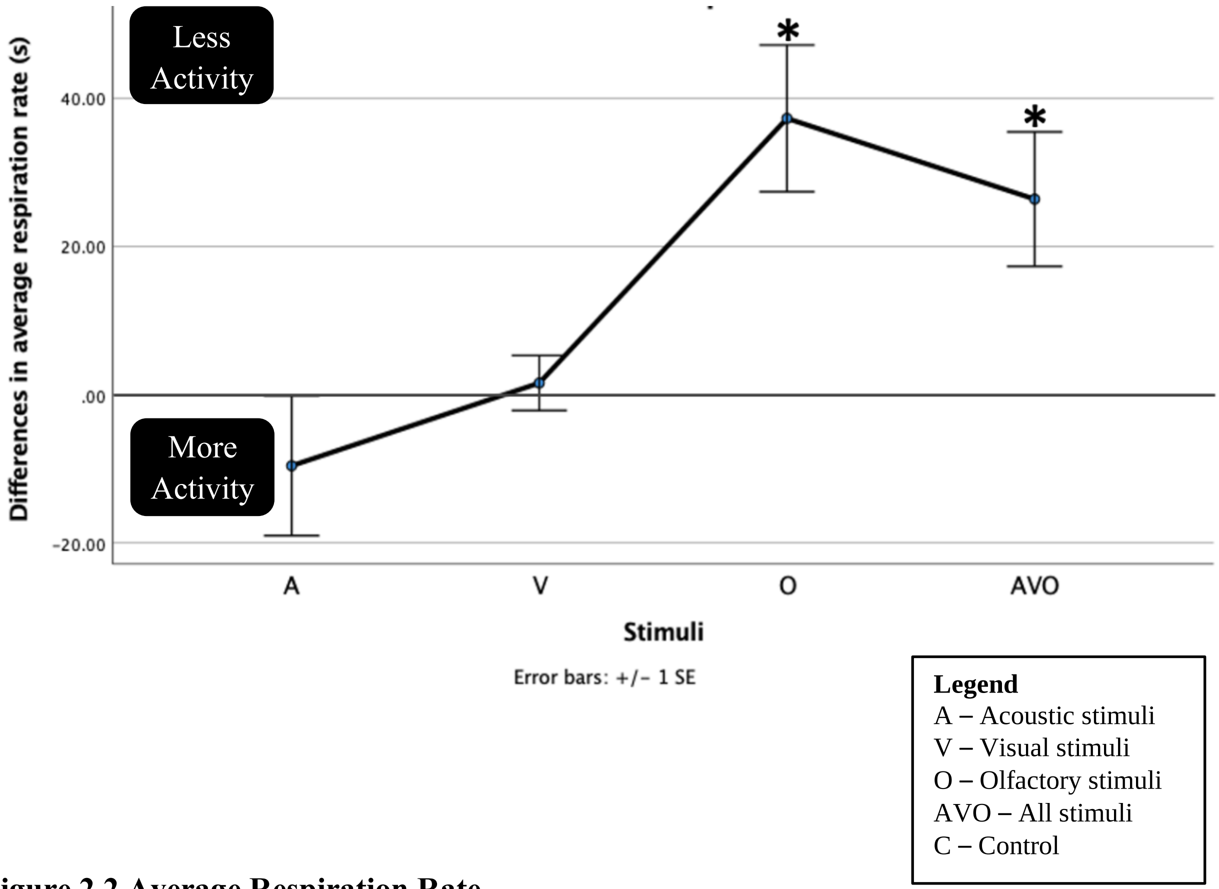


Figure 2.2 Average Respiration Rate

The bolded zero line on the graph is a control baseline representing that the stimuli had no effect on average respiration rate. The results show that stimuli had a significant decreasing effect on the overall average respiration rate ($F_{2.937,55.800}=6.859$, $p<0.001$) and when presented with olfactory stimulus ($p=0.001$) and the combined AVO stimulus ($p=0.009$).

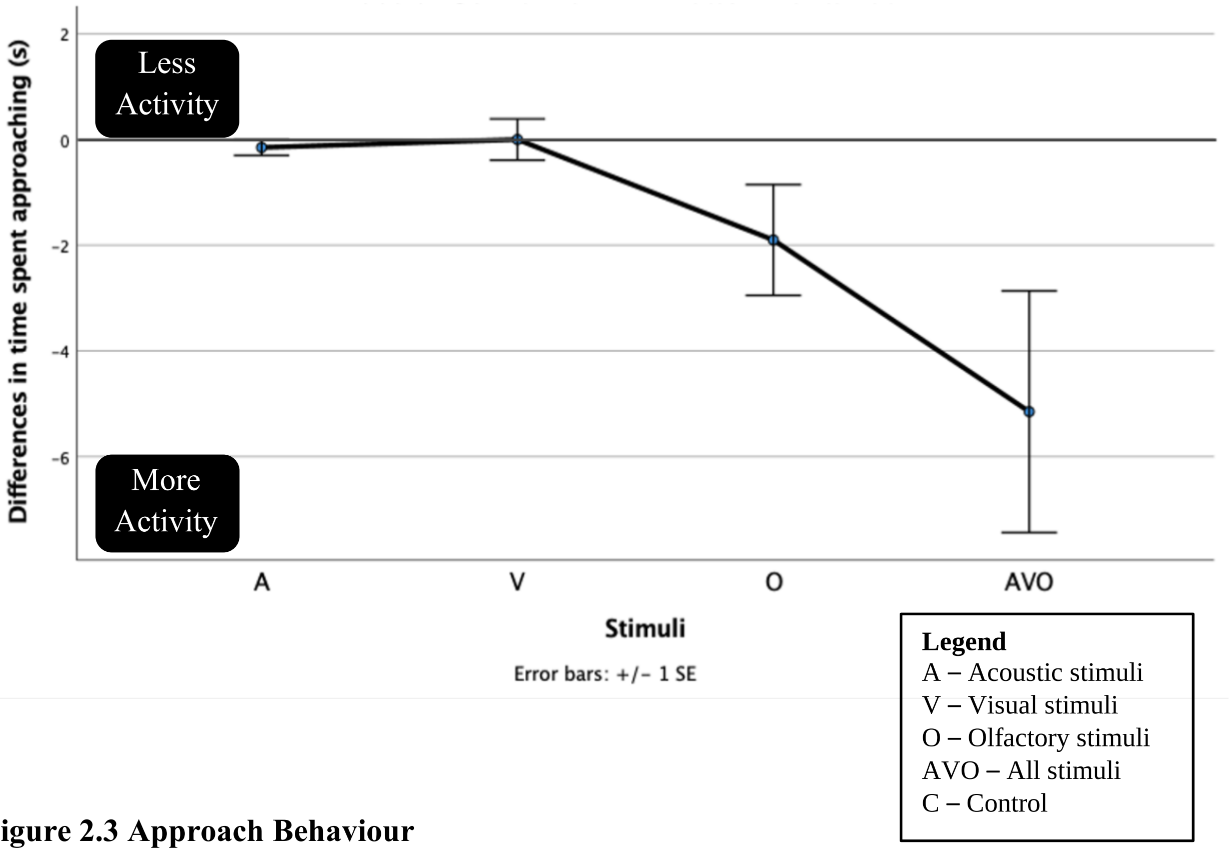


Figure 2.3 Approach Behaviour
The bolded zero line on the graph is a control baseline representing that the stimuli had no effect on approach behaviour. There was no significant effect on the overall time spent approaching ($F_{1.436,27.283}$=4.410, *p*=0.033).

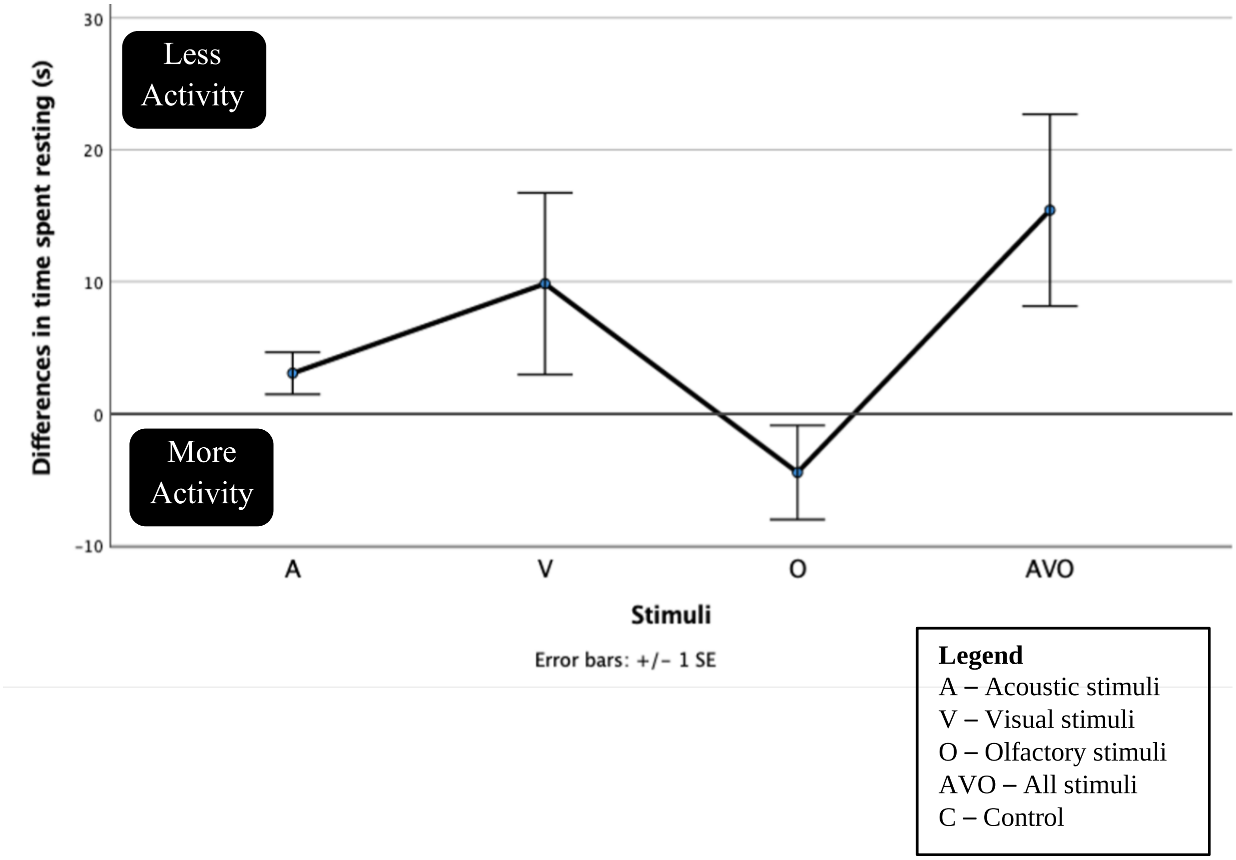


Figure 2.4 Rest Behaviour
The bolded zero line on the graph is a control baseline representing that the stimuli had no effect on resting behaviour. The was no significant effect on time spent resting ($F_{2.421,43.572}=3.080$, *p*=0.047).

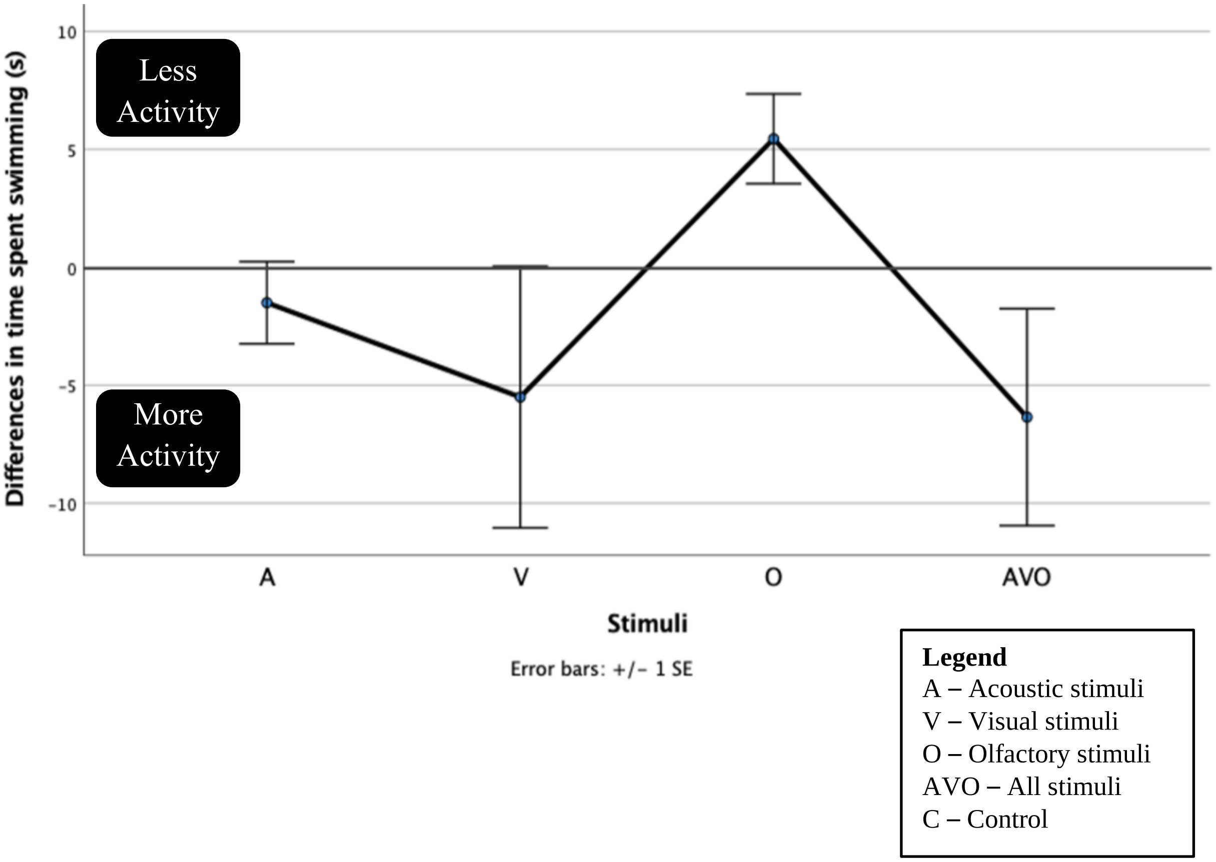


Figure 2.5 Swim Behaviour
The bolded zero line on the graph is a control baseline representing that the stimuli had no effect on swimming behaviour. The results show no significant effect on the time spent swimming ($F_{2.155,40.179}=1.874$, $p=0.165$).

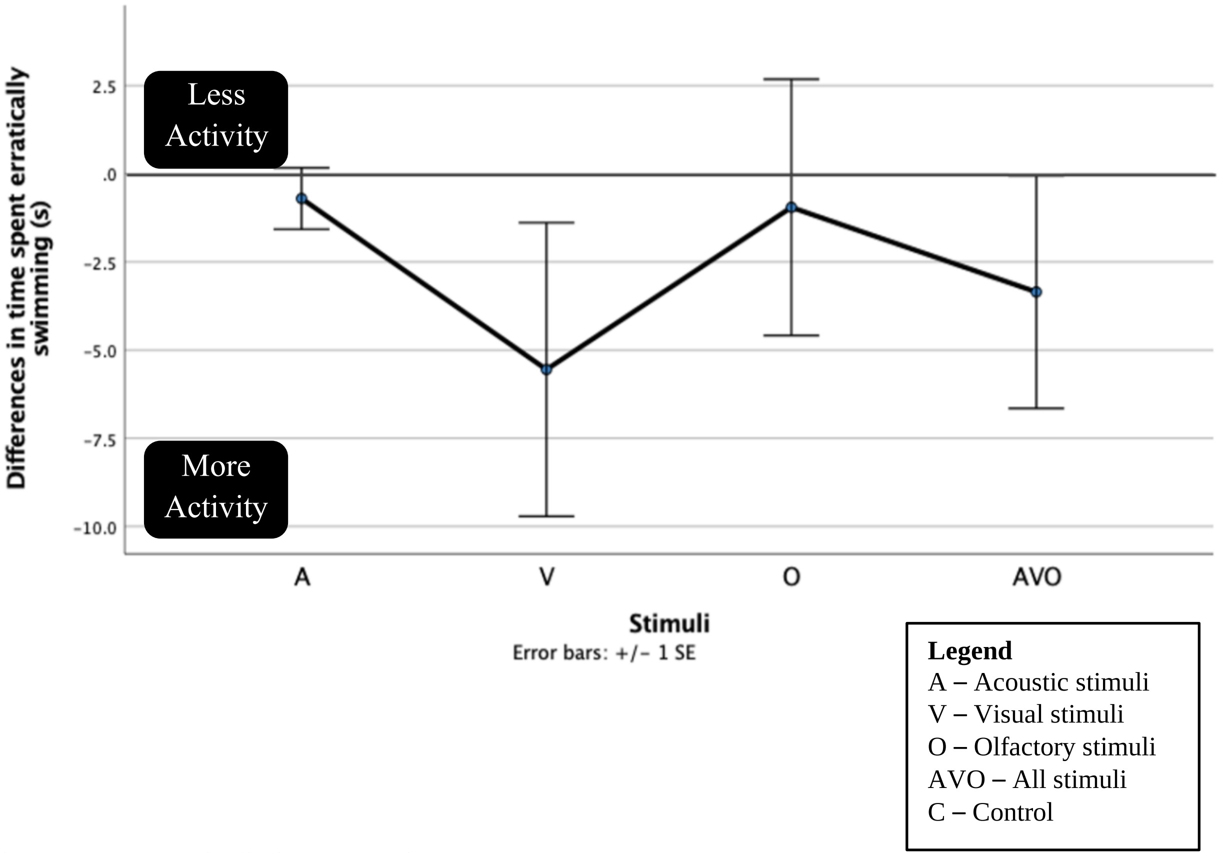


Figure 2.6 Erratic Swim Behaviour
The bolded zero line on the graph is a control baseline representing that the stimuli had no effect on erratic swimming behaviour. The results show no significant effect on the time exhibiting erratic swimming behaviour ($F_{1.870,35.530}=0.962$, $p=0.387$).

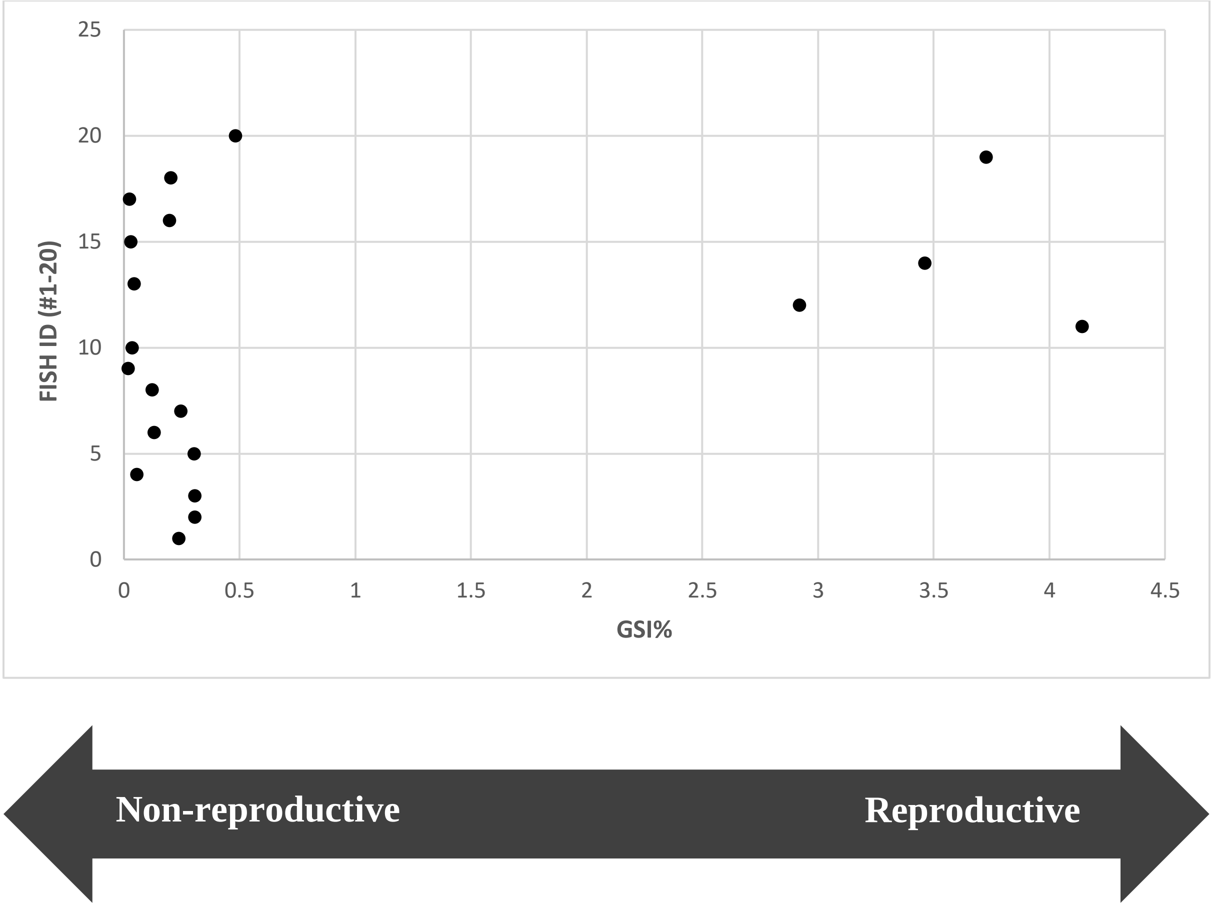
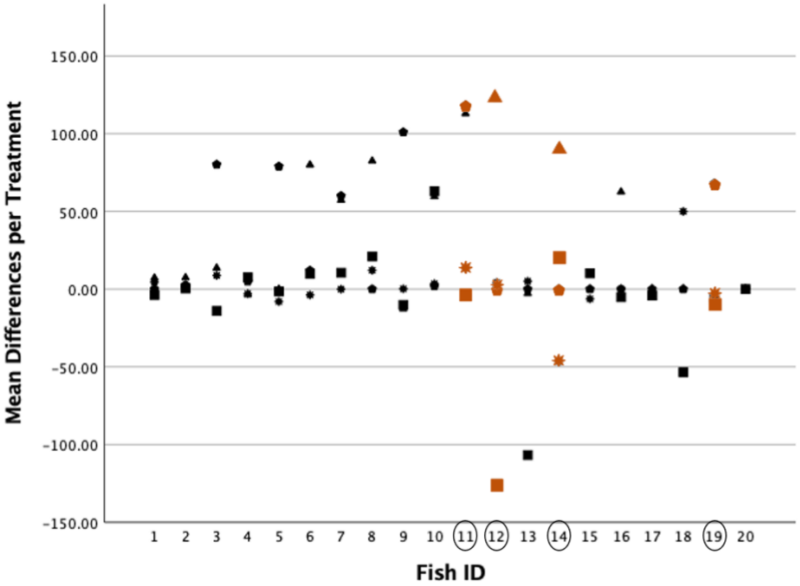


Figure 2.7 Gonadosomatic Index (GSI) of Round Gobies

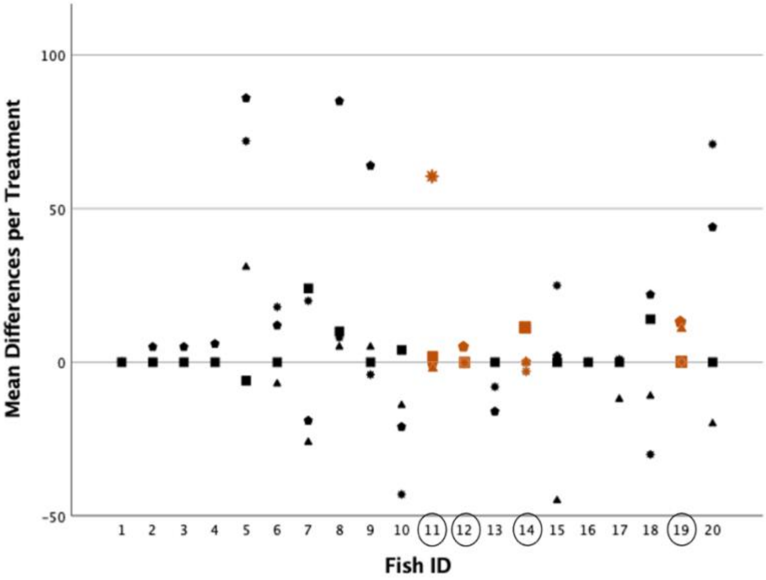
The gonadosomatic index (GSI) was calculated for each fish to determine reproductive maturity. The calculations revealed that Fish #11 (GSI= 4.145%), #12 (2.919%), #14 (3.462%) and #19 (3.726%) were reproductive while all other fish were non reproductive (GSI<1%).

Legend
Orange = Reproductive
Black = Non-Reproductive

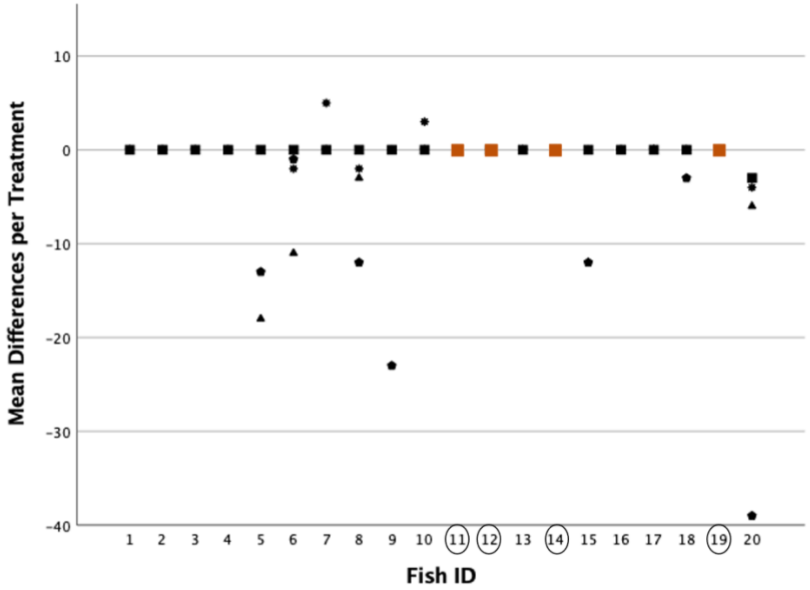
A



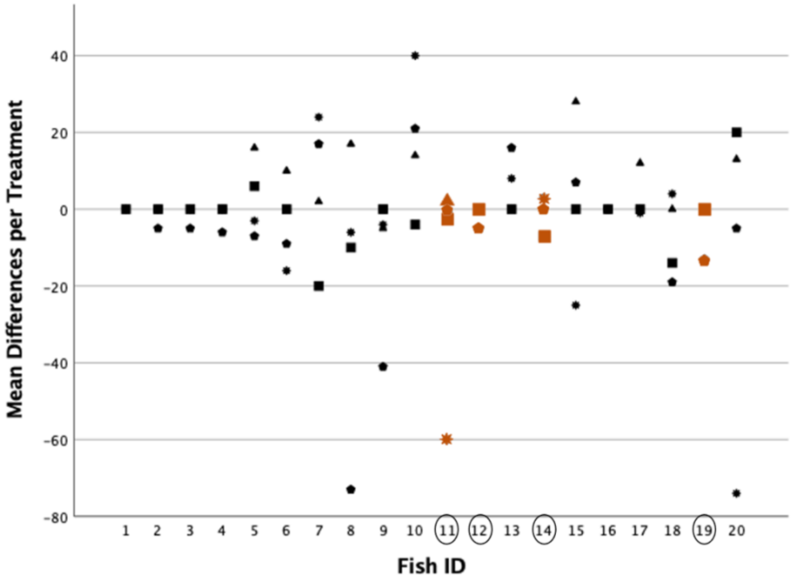
B



C


Mean Differences per Treatment
10
0
-10
-20
-30
-40
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
Fish ID

D


Mean Differences per Treatment
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0
-20
-40
-60
-80
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
Fish ID

E

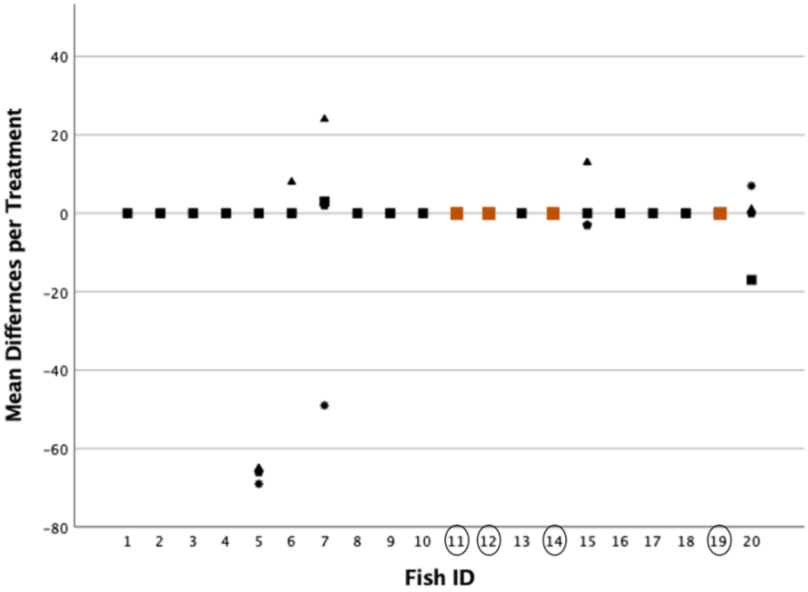

Figure 2.8 Reproductive Males vs. Non-Reproductive Males
The graph compares the average time exhibiting quantified behaviours of reproductive males round gobies (orange) to non-reproductive males (black) for each treatment. Only four reproductive males (Fish #11, 12, 14, and 19) were identified within the sample. Comparing both reproductive morphs revealed that average respiration rate (A) and the time spent approaching (B), resting (C), swimming. (D), and erratically swimming (E) resting in reproductive males does not vary and falls within the distribution of behavioural responses of non-reproductive males.

CHAPTER 3

AUDITORY REGENERATION IN BLACK BULLHEAD (*AMEIURUS MELAS*) FOLLOWING BOAT NOISE EXPOSURE

Introduction

Fish rely on sound as a sensory modality for homing orientation, predator-prey detection (Fay & Popper, 2000; Casper et al., 2013; Ladich & Fay, 2013), reproduction (Rollo et al., 2007) and territory defense (Fay, 2009; Kasumyan, 2009; Slabbekoorn et al., 2010) but increasing levels of anthropogenic noise may cause disruptions and mask important acoustic signalling (Codarin et al., 2009; Popper & Hastings, 2009; Casper et al., 2013; Simpson et al., 2010). Globally, the most dominant source of underwater anthropogenic noise is ship noise, which propagates efficiently at low frequencies underwater (Richardson & Würsig, 1997; Vasconcelos et al., 2007) and can overlap with fish communication signals (Radford et al., 2014). While there is evidence that noise from ships and recreational boats can impact marine fish (Weilgart, 2007; Heide-Jorgenson et al., 2013; Dyndo et al., 2015), the impact of this noise source in freshwater habitats is poorly understood (Popper, 2003; Slabbekoorn et al., 2010; Mickle & Higgs 2018), therefore furthering the need for more research on noise exposure in freshwater fish species.

Sound is ultimately transduced into neural impulses in the inner ear of fish, which is composed of three semicircular canals and three inner ear organs: saccule, utricle and the lagena. The inner ear also contains sensory epithelium (macula) and the otolith (Popper et al., 2003; Smith et al., 2011; Monroe & Smith, 2015). The otolithic end organs have both vestibular and auditory functions, depending on the specific macula, (Saidel et al., 1990; Smith et al., 2006; Popper et al., 2003; Monroe & Smith, 2015) and each macula has ciliary bundles that are responsible for sensory transduction (Fettiplace & Ricci, 2006). The sensory organs contain ciliary bundles of hair cells that are essential for hearing and can be damaged when exposed to high intensity sound (McCauley et al., 2003; Popper, 2003), which may induce temporary threshold shifts and lead to hearing loss (Hastings et al., 1996; Smith et al., 2004; Smith et al., 2011; Monroe & Smith, 2015; Simpson et al., 2010; Mickle & Higgs, 2018). Additionally, high intensity sounds can cause ruptures in the swim bladders of fish and other stressors such as increases in cortisol levels (stress hormone), increased metabolic-ventilation rate, altered foraging efficiency, induce avoidance behaviour and increased startle and sheltering response (Mickle & Higgs, 2018; Pieniazek et al., 2020) Anthropogenic noise can negatively impact many fish species but fish can also regenerate ciliary bundles following noise exposure (Popper & Hoxter, 1984; Corwin & Oberholtzer, 1997). Fish can repair their ciliary bundles throughout their lives and therefore can potentially minimize the impact of noise (Corwin et al., 1988; Smith et al., 2006). The post-embryonic proliferation of sensory ciliary bundles may play a role in self-repair mechanisms following noise exposure (Corwin & Oberholtzer, 1997) but for many species it remains unclear how long this regeneration can take under realistic levels of anthropogenic noise exposure. To better model the long-term hypothesized effects of noise on fish, a better understanding of the time course of regeneration in a range of species is needed, as fish in many areas will only be exposed to high noise intermittently.

In the current study, we assessed the role of anthropogenic boat noise, played at different sound levels, on ciliary counts and regeneration in black bullhead (*Ameiurus melas*). Black bullhead are members of the Ostariophysi with well-known specializations for enhanced hearing capabilities (Ladich & Popper, 2004). Black bullhead are good models for potential hearing damage due to the presence of Weberian ossicles, an accessory hearing structure, which connect and transmit vibrations from the swim bladder (air-filled chambers) to the inner ear allowing them to detect sound stimuli at high frequencies and making them more prone to ciliary bundle damage following high intensity sound exposure, compared to other freshwater fish species (Ladich & Wysocki, 2003; Lechner & Ladich, 2008; Casper et al., 2013). Two separate experiments were conducted. Experiment 1 aimed to determine whether black bullhead could regenerate ciliary bundles following 1.5 hours of anthropogenic boat noise exposure at 170 dB re 1 µPa. Experiment 2 aimed to determine the effects of noise on ciliary bundles composition and how regeneration varies by exposing fish to anthropogenic boat noise at various sound intensities (160, 165, 170 and 175 dB re 1 µPa) for 2 hours. I hypothesized that both experiments will show moderate damage to ciliary bundles with the most damage caused by 170 and 175 dB re 1 µPa. I predict that regeneration will occur after exposure to each sound level and that a longer recover time will be needed as sound intensity increases.

Methods

General Methods

All black bullhead (Total Length: 10-15cm) used for both studies were collected from Todd Leady Environmental Corporation in Harrow, Essex County, Ontario (42°01'14.5"N 83°00'04.1"W). All fish were kept in a housing tank (48” x 18.5” x 21”) in the Central Animal Care Facility at the University of Windsor and maintained following Canadian Council on Animal Care (CCAC) guidelines with the following conditions: temperature of 21℃, a pH of 6.5-7.5 and a 12:12 light/dark cycle. To simulate their natural dark environment, tanks were covered with a black garbage bag. Experimental trials for both studies took place in an adjacent room in plastic experimental tanks (53 x 36 x 35cm). To reduce sound reverberation effects in glass tanks (Parvulescu, 1964), plastic tanks were used. The tanks were placed on top of Styrofoam to reduce vibrations from the ground during experiments. Experimental tanks were equipped with a filter, air stones, and shelters; however, both the filter and bubblers were turned off during experimentation. Sounds for noise exposure were played using an MP3 player (Sony Walkman NWZ-E464) connected to an underwater speaker (Electro-Voice UW-30) and an amplifier (Pioneer Max Power 400W) which was powered by a rechargeable battery (Leoch 12 V) for noise trials (Mickle et al., 2019). The sound file used for experiments was a recording of a recreational vessel with a four-stroke outboard motor obtained by a hydrophone (Loggerhead Instruments, Model # HTI-96-Min/3V/Exp/LED) which was placed about 4m from a boat launch in an embayment on the Detroit River (LaSalle, Ontario, Canada at a depth of roughly 2m (Mickle et al., 2019). The boat noise sound file had a significant amount of energy up to 4000 Hz, which approximates the known hearing range of the fish family Ictaluridae (Weiss et al., 1969), and a flat power spectrum up to 16 kHz (Fig. 3.1). Background noise levels were previously recorded by Mickle et al. (2019), which used the same experimental set-up and were consistently below 120 dB re 1 µPa. Sound levels were measured during noise presentation along 12 positions of the tank (each covering an area of 10"x 8") using a hydrophone (inter Ocean system inc.- Acoustic Calibration and System Model 902) to establish a range of sound levels within the tank, which were then averaged to provide values of 160, 165, 170 and 175 dB re 1 µPa during experimental trials. Sound levels were also measured in one location prior to each treatment, to ensure consistency in exposure levels before the start of each experiment.

In the current study, two separate experiments were conducted. Experiment 1 was conducted in 2018 and investigated regeneration of ciliary bundles following noise exposure for black bullhead. Experiment 2 was conducted in 2019 as a follow-up study to investigate how ciliary bundle regeneration varies with exposure to different sound intensities. Control trials for both studies replicated experimental conditions, however, the fish were not exposed to any boat noise. In total, 52 fish were exposed to boat noise and were chosen at specific timepoints to be euthanized using 2-phenoxyethanol (1ml of 2-phenoxyethanol per 2L of dechlorinated water) (Mickle et al., 2019) and then decapitated using a scalpel. Bullhead heads were placed in a jar containing paraformaldehyde (4%) to preserve the tissues prior to dissection (Mickle et al., 2019). Under a dissecting microscope (Leica S6D), saccules were removed from the head and then otoliths were removed from ears to expose the sensory maculae (Mickle et al., 2019). To visualize ciliary bundles, the epithelia of each fish were stained using a mixture of 12.5µL of Oregon green phalloidin (Life Technologies) and 200µL of phosphate buffer and left in a mixing well for 20 minutes in a dark drawer (Higgs et al., 2002). After the elapsed time, the stained epithelia were placed on microscope slides (Fisherbrand) and a drop of Fluoroshield with DAPI (Sigma) was placed onto the tissue. Using the Leica S6D dissecting microscope the samples were oriented so that the opening that was made to remove the otolith was facing upwards and a microscope cover glass (UtiliDent) was placed over the tissues to flatten them for better visualization of ciliary bundles. A thin coat of nail polish was added to the edges of the cover slip as an adhesive. All slides were placed in a slide box and refrigerated, and images were taken within 4 days to avoid fading. Ciliary bundles were visualized by taking images using a fluorescent microscope (LEICA M205 FA). Images were captured using the LASX software at different magnifications to get a clear image of the entire saccule. Three identical boxes measuring 200 x 200 μm for both studies were placed onto the image of the saccules in three different regions of the sensory epithelium using Adobe Photoshop CS6. The boxes were placed at the top, middle and bottom regions of the saccule and had similar density of ciliary bundles. Creating boxes in three different regions allowed quantification of possible damage in three different areas of the saccule to determine whether noise exposure trials caused equal morphological damage or if certain regions of the saccule showed more damage than other regions. Using Image J (NIH IMAGE), ciliary bundles were then counted and recorded from each of the three boxes. Damage caused from dissection was notably different from ciliary bundle loss, as dissection damage often appeared as a tear while ciliary loss appeared as dark spots. (Hastings et al., 1996).

Experiment 1

A total of 32 black bullhead were used for experimental trial and control trials. Three experimental trials (n=24) and two control trials (n=8) were conducted. Each noise trial exposed eight black bullhead to 170 dB re 1 μPa of boat noise for 1.5 hours and control trials exposed four black bullhead to a "quiet period" of 1.5 hours. At the end of each noise experiment, one fish was removed from the experimental tank for dissection of the saccules at each of the following timepoints: 0, 24, 48, 72, 96, 168, 336 and 504 hours after initial noise exposure (Fig. 3.2A and 3.2B). Control trials followed a similar schedule with the removal of fish for dissections at the following timepoints: 0, 48, 96 and 336 hours after the "quiet period".

Experiment 2

A total of 34 black bullhead were used for experimental trial and control trials. Four experimental trials (n=28) and two control trials (n=6) were conducted. A group of seven fish were assigned to each sound intensity, 160, 165, 170 and 175 dB re 1 µPa, and were exposed to 2 hours of boat noise at their assigned sound intensity. Control trials exposed four fish to a 2-hour "quiet period". At the end of each noise experiment one fish was removed from the experimental tank for dissection of the saccules at each of the following timepoints: 0, 24, 48, 72, 96, 168 and 192 hours after initial noise exposure (Fig. 3.2C and 3.2D). Control trials followed a similar schedule with removal fish for dissections at the following timepoints: 0, 72, 192 hours after the "quiet period" (Fig. 3.2C & 3.2D).

Statistical Analysis

Using the statistical analysis program, SPSS (version 23, IBM SPSS Statistics, Chicago, IL) a univariate analysis of variance (ANOVA) was performed for both experiments, as the data was normally distributed. Dependent and independent variables were defined as ciliary bundle counts and treatment, respectively. A Tukey post-hoc test was then used to make pairwise comparisons where differences occurred. A significance level of $\alpha = 0.05$ was used for all statistical tests. Statistical analysis tested for the effect of time and noise on ciliary bundle counts individually and as an interaction effect for both experiments. The analysis was done on the three shared timepoints of the control trials and the experimental trials, which were at 0 hours, 48 hours, and 96 hours following noise exposure for Experiment 1, and 0 hours, 72 hours, and 192 following noise exposure for Experiment 2. To directly compare the time points of the control trials to noise experiments, only the three time points for each experiment mentioned above were used in the analysis. It was determined that there was no significant difference between the ciliary bundle counts of the three regions/boxes (Exp. 1: p=0.487; Exp. 2: p=0.690), therefore, all data from all three sections were used in the statistical tests.

Results

Experiment 1:

The analysis was run on the three shared timepoints (0, 48, and 95 hours) of the control and experimental trials which determined that there was a significant main effect of time ($F_{2,39}$=10.975, $p<0.05$) on ciliary bundle counts, no significant effect of noise ($F_{1,39}$ = 3.987, p=0.053) on the number of ciliary bundles and no interaction effect of time and treatment ($F_{2,39}$= 1.572, p=0.230). Post-hoc testing for the effect of time on ciliary bundle counts revealed that differences occurred only at 1.5 hours ($p<0.05$; Fig. 3.3) but by 48 hours (p=0.104) and 96 hours (p=0.104) there was no significant effect of time on ciliary bundle density indicating that regeneration of ciliary bundles took place right after noise exposure and ceased at 48 hours. An increase in bundle counts was observed immediately following exposure and gradually began to increase in number through 48 hours (p=0.104) up until 96 hours (p=0.104) where ciliary counts start to level off and return to control levels (Fig. 3.3). No permanent damage was seen at this intensity for this duration.

Experiment 2:

There was a significant effect of time ($F_{8,71}$=24.968, $p<0.05$) and noise ($F_{3,71}$= 96.545, $p<0.05$) both separately and as an interaction effect ($F_{18,71}$=3.544, $p<0.05$) on ciliary bundle counts. For each sound intensity (160, 165, 170, 175 dB re 1 µPa), three time points of (0 hours, 72 hours, and 192 hours) were compared to the control (Fig. 3.4). Sound levels of 160 and 165 dB re 1 μPa did not have an effect on ciliary bundles counts and were not significant for 0 hours (160 dB re 1 μPa: p=0.108; 165 dB re 1 μPa: p=0.817), 72 hours (160 dB re 1 μPa: p=0.511; 165 dB re 1 μPa: p=0.168), and 192 hours (160 and 165 dB re 1 μPa: p=1.000) (Fig. 3.5). Noise exposure at 170 dB re 1 μPa showed a significant effect on ciliary bundles at 0 (p<0.05) and 72 hours (p<0.05), however, they returned to control levels by 192 hours (p=0.894) (Fig. 3.5). There was a gradual increase in ciliary bundle counts from 0 to 192 hours indicating that they were potentially regenerating during this time and ceased regeneration by 192 hours (p=0.894) (Fig. 3.5). Noise played at 175 dB re 1 μPa resulted in the ciliary counts being significantly lower than other intensities and was significant for all time points; 0 hours (p<0.05), 72 hours (p<0.05) and 192 hours (p<0.05) indicating that louder noise intensities as well as how long the noise is played for can prolong the regeneration time and ultimately take longer to repair ciliary bundles (Fig. 3.5).

Discussion

The aim of the current study was to investigate the time course of regeneration of ciliary bundles following boat noise exposure at various sound levels. The study conducted by Mickle et al. (2019) was used as a framework to develop Experiment 1 and 2. Mickle et al. (2019) exposed bullhead to boat noise for short (1 hour) and long (24 hours) periods at 160 dB re 1 μPa and 170 dB re 1 μPa and found that damage to the saccule occurred when boat noise was played at 170 dB re 1 μPa and no damage was seen at 160 dB re 1 μPa. This is consistent to the findings of Experiment 1 and 2 where both experiments show that damage starts to occur at 170 dB re 1 μPa. Exposing bullhead to higher sound levels than what was tested here could cause even more damage which could lead to longer regeneration time or hearing loss. Noise played at 175 dB re 1 μPa caused the most damage and although ciliary counts showed signs of regeneration, the ciliary counts never reached control values. It is possible that the timeline of our study was not long enough, and more recovery time was needed to see full regeneration. However, even if louder sound levels are tested there could be a point where regeneration will not be possible due to the morphological damage to the sensory epithelia and could lead to hearing loss. Boat noise can be a threat to black bullhead in terms of hearing loss which may affect their survival if they aren't able to localize calls from conspecifics or respond appropriately to other important acoustic signals within their environment.

The two driving forces causing loss of ciliary bundles are the increase of anthropogenic boat noise sound levels and the amount of time exposed to this noise. Globally, the presence of shipping boats and recreational boats in aquatic environments is increasing (Slabbekoorn et al., 2010; Simpson et al., 2010), thus sound pollution generated by these sources are in turn growing, representing a larger concern for fish. The common goldfish *(Carassius auratus)* and fathead minnow (*Pimephales promelas)*, both with enhanced hearing abilities, have both been shown in past research to experience shifts in hearing threshold (Scholik & Yan, 2001; Smith et al., 2004; Smith et al., 2006; Smith et al., 2011). The common goldfish exhibited a loss of ciliary bundles when exposed to boat noise, with subsequent regeneration of ciliary bundles and recovered its hearing abilities (Smith et al., 2004; Smith et al., 2006; Smith et al., 2011) The audio brainstem response (ABR) technique was used in the Scholik & Yan (2001) study where they exposed white noise to the fathead minnow (*Pimephales promelas*) at 142 dB re 1 μPa and determined significant changes to their auditory threshold. In contrast, Scholik & Yan (2002) exposed the bluegill sunfish (*Lepomis macrochirus*), a species without enhanced hearing sensitivities, to the same white noise at the same intensity as Scholik & Yan (2001) at multiple frequencies and different durations and determined that there was no significant difference on auditory thresholds for all frequencies tested. Damage to the sensory epithelium and shifts in auditory threshold resulting from noise exposure seems to be species dependent. Hearing abilities of the species should be considered when assessing the effects on noise on the inner fish ear. on the species as well as the sound intensity and duration of the noise.

The ability of fish to regenerate sensory ciliary bundles has sparked an interest in identifying a mechanism for sensory hair cell proliferation following acoustic damage (Monroe & Smith., 2015) however, these mechanisms are still not well understood (Higgs, 2020). Post-embryonic cell proliferation continues throughout the adult life of a fish and can either slow down or stop as fish age (Popper & Hoxter, 1984). The regenerated ciliary bundles are beneficial to the survival of fish especially those with enhanced hearing which can be affected by various sound intensities since they can hear a wider range of frequencies. The ability to hear biologically relevant signals is pertinent to a fish's performance and survival within their environment, however there are still gaps in research that have yet to address the noise levels needed to cause permanent damage. While it is clear that boat noise can cause damage upon short-term intense exposure (Popper, 2003), evidence has shown that fish can avoid the noise source and move to quieter areas (Ona & Godø, 1990; Holles et al., 2013; De Robertis & Handegard, 2013) suggesting that damage from noise exposure may be short-lived. Anthropogenic noise can mask important vocalizations needed for reproductive events such as spawning and courtship interactions (Slabbekoorn et al., 2010). The ability to hear important vocalizations is essential for reproduction (Maruska et al., 2012; Amorim et al., 2015), however if fish have impaired hearing and even hearing loss due to damage from noise exposure, even for a short period of time, the ability to assess mates and reproduce effectively may be compromised (Radford et al., 2014). Fish that have experienced damage to ciliary bundles, even for short durations of time, may not be able to detect auditory cues related to predator or prey detection (Purser & Radford, 2011; Simpson et al., 2010). However, this may also be species specific as the increased levels of vocalizations such as background noise or boat noise has no effect on the mating calls of round goby (*Neogobius melanostomus*), a highly invasive species with limited hearing range (Higgs & Humphrey, 2019).

Future studies should follow up by assessing the hearing abilities of black bullhead (using techniques such as Auditory Brainstem Response (ABR)) during the times associated with lower ciliary bundle counts as a result of noise exposure, to determine the presence of a potential auditory threshold shift. Further research is also needed to determine if permanent sensory damage can occur in black bullhead by subjecting bullhead to higher sound intensities for longer periods of time. Future studies can also consider providing a longer recovery period when exposed to 175 dB re 1 µPa of boat noise to determine if ciliary counts can reach control levels. Although anthropogenic noise exposure causes decreased counts in ciliary bundles in bullhead, the experimental setup represents an artificial scenario, whereby black bullhead are constrained close to the sound source (Smith & Monroe, 2016). Therefore, field studies should be conducted to understand how black bullhead respond to high intensity sound in their natural environments (Smith & Monroe, 2016; Pieniazek et al., 2020). The fish used in the current study did not differ dramatically in size, therefore, future research should look at the different stages of development and investigate how this affects regenerative capabilities. Future research should also study the effects of anthropogenic noise on fish that lack hearing specializations to investigate if damage to sensory ciliary bundles is possible at different sound intensities.

Ambient sound levels are on the rise, approximately increasing at a rate of 3.3 dB re 1 μPa per decade (Frisk, 2012), therefore, research investigating how increasing intensities of anthropogenic noise affects fish is important to understand the range of human impact on aquatic life. While there are studies looking at the impacts of anthropogenic noise on fish in marine environments, there is less known about how it can affect freshwater fish (Slabbekoorn et al., 2010; Mickle & Higgs, 2018). It is vital to protect the biodiversity of fish by implementing protocols to mitigate anthropogenic noise in freshwater ecosystems, especially considering there are high human populations near these environments (Dudgeon et al., 2005). Perhaps in high traffic aquatic habitats where anthropogenic noise levels are high, quiet refuges (areas that prohibit loud vessels or noises at high sound intensities) can be implemented to allow for fishes to either avoid noisy environments or recuperate from noise exposure damage. It is reasonable to assume that fish possessing a Weberian apparatus, like the black bullhead, are more sensitive to impulsive sound sources, thus damage to ciliary bundles likely has a larger impact on this fish species (Slabbekoorn et al., 2010; Ladich & Fay, 2013). Hence, it is important to protect fish species from anthropogenic noise exposure, especially fish with specialized hearing sensitives.

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FIGURES

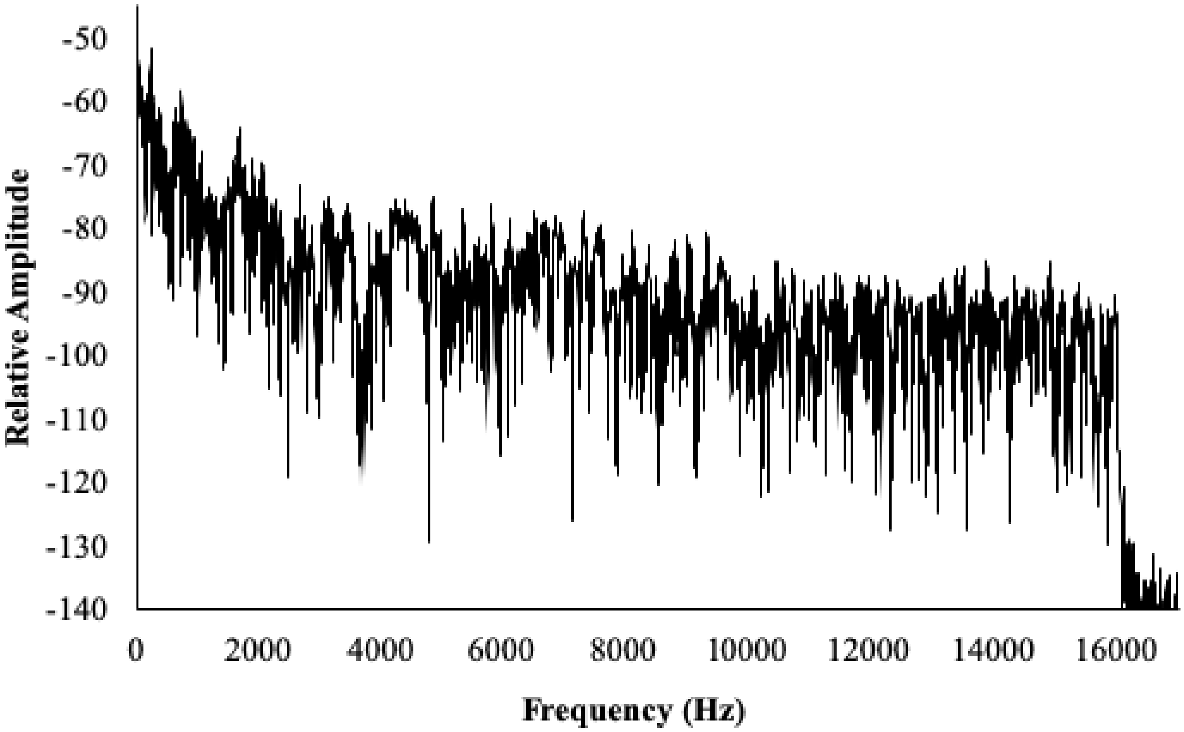

Figure 3.1 Frequency Analysis of Boat Noise File

A frequency analysis of the boat noise file from the field recorded with a hydrophone showcasing a frequency range of 100 - 16000Hz.

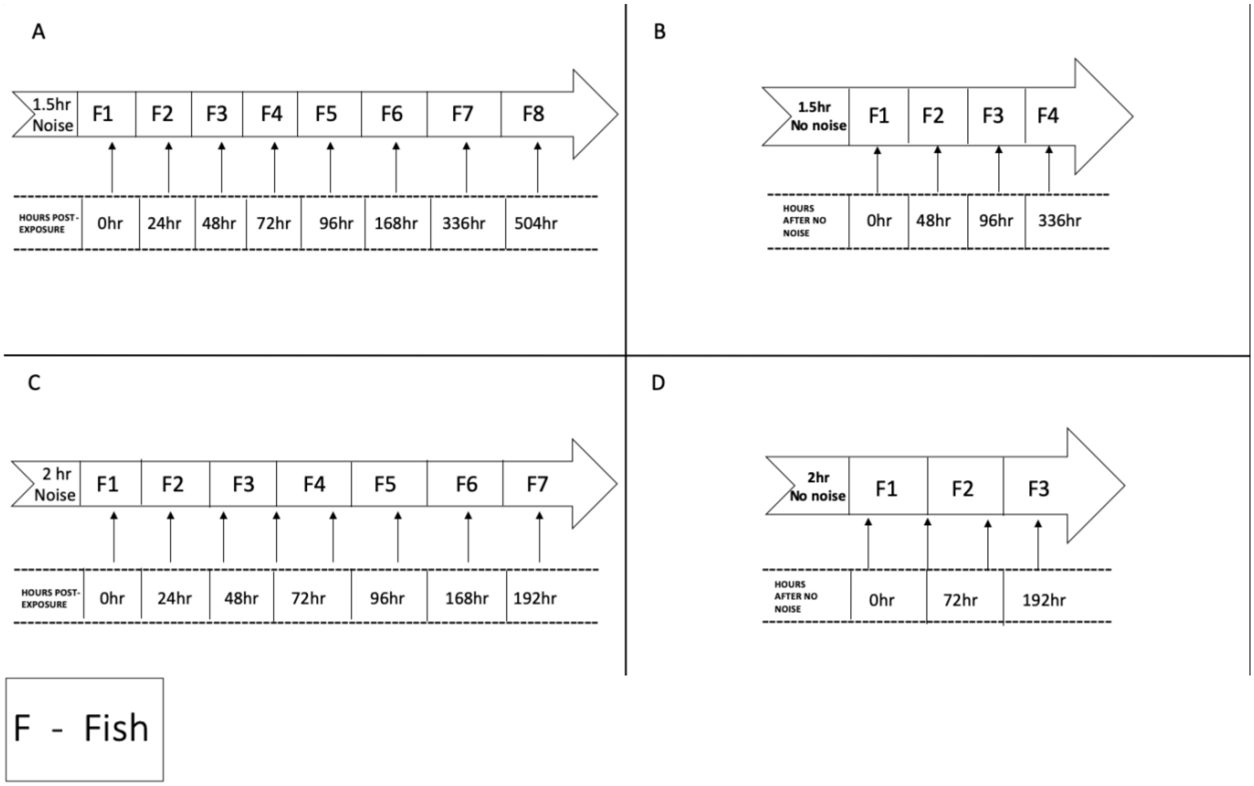

Figure 3.2 Experimental Timeline

Following noise exposure (experimental) and no noise exposure (control), fish were selected for sacrifice at specific time point following the post-exposure sacrifice schedule A) and B) for Experiment 1 and C) and D) for Experiment 2.

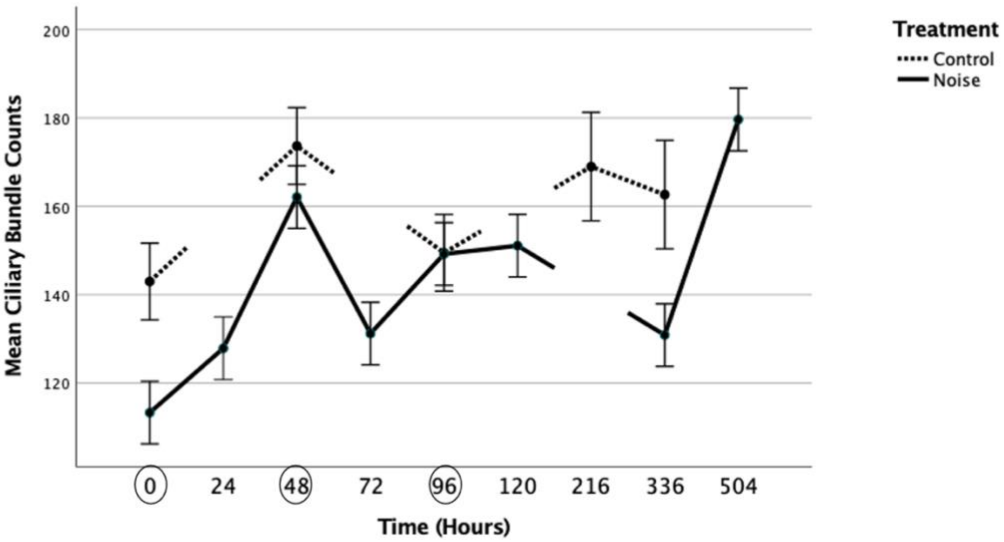

Figure 3.3 Experiment 1: Average Ciliary Bundle Counts Over Time

The averaged hair cell counts of Experiment 1 show little change over time in control conditions but decrease immediately after noise exposure followed by a gradual increase to control levels. (Effect of time: $F_{2,83}$= 10.380, $p<0.05$) Three treatment timepoints (0, 48, and 96) were compared to control time points in statistical testing where a significant decrease in ciliary bundle counts was seen immediately following noise exposure at 0 hours ($p<0.005$) and returned to control levels by 48 hours post noise exposure ($p=0.104$). (Error bars represent SE).

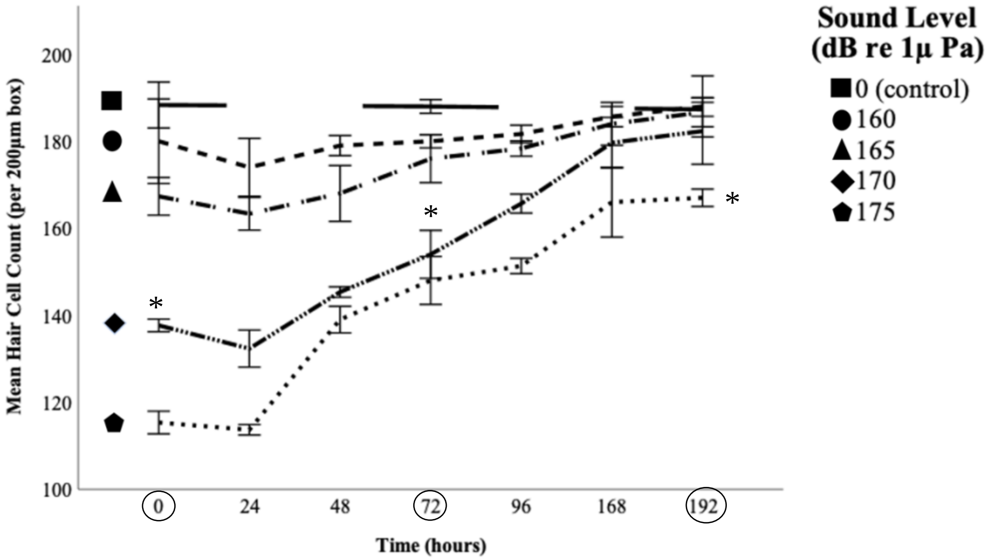

Figure 3.4 Experiment 2: Regeneration Trends in Ciliary Bundles Counts
Mean sensory hair cell counts quantified from saccules from increasing regeneration times for study 2. The graph depicts sensory hair cell counts from ears exposed to no noise, 160, 165, 170 and 175 dB re 1 µPa of boat noise. (Error Bars: +/- 1 S.E).

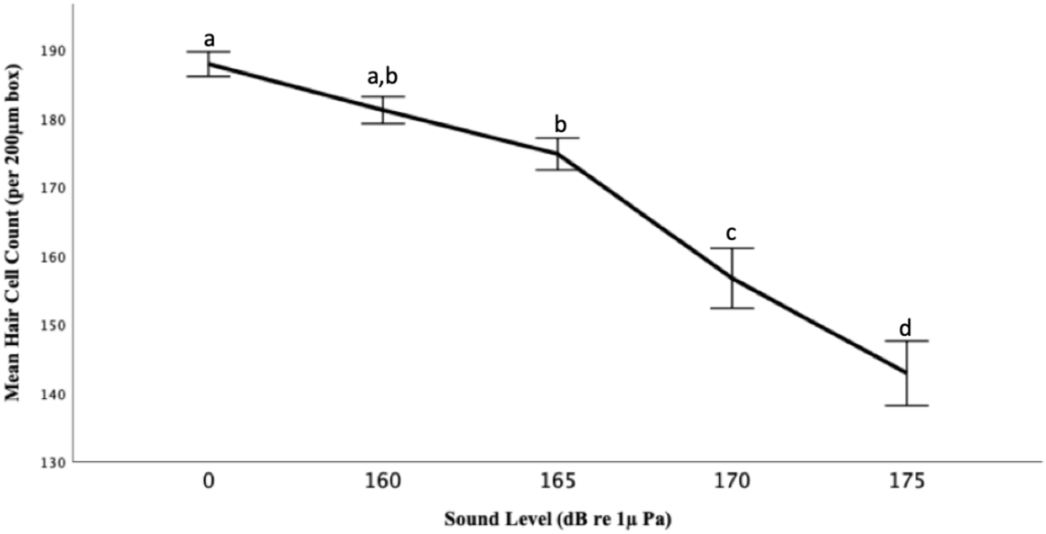

Figure 3.5 Experiment 2: Average Ciliary Bundle Counts at Multiple Intensity
Mean sensory hair cell counts (per 200x200µm box) for Experiment 2 when bullhead were exposed to 160, 165, 170 and 175 dB re 1 µPa of boat noise. A no-noise control is used to compare noise exposed bullhead with the control and to detect if bullhead are affected by ambient sound levels. Different letters indicate significant differences in sensory hair cell counts at the *p*=0.05 level. (Error Bars: +/- 1 S.E). The sound level 0 denoted on the x-axis indicates no playback but some ambient noise.

CHAPTER 4

CONCLUSIONS AND RECOMMENDATIONS

Summary

The main objective of my thesis was to look at the effect of various types of stimuli on behaviour and physiology in two freshwater fish species found in the Great Lakes. Chapter 2 focused on the effect of various types of stimuli on behaviour in the round goby and chapter 3 focused on the effect of anthropogenic noise on the inner ear morphology in black bullhead. Chapter 2 investigated the synergistic responses of the round goby to multiple stimuli through the presentation of unimodal and multimodal stimuli and how reproductive status can influence this response. Synergistic responses to multimodal signals can occur and can improve the localization and detection of signals in fish communication ensuring that the intended message is interpreted correctly (Partan & Marler, 2005; Kasurak et al., 2012). Taking an integrative approach in multimodal signalling experiments can reveal what synergistic responses look like in other fish species. In chapter 2, I conducted behaviour experiments where I presented acoustic, visual, and olfactory stimuli individually and simultaneously. The time spent exhibiting behaviours such as approaches to stimuli, resting, swimming, and erratic swimming were quantified for each experiment. The results determined that the average respiration rate decreased in response to multimodal stimuli showing evidence that a synergistic response may be occurring.

The synergistic response was tested against an additive response which determined that the response to the multimodal stimuli was greater than sum of responses to individual stimuli. The time spent approaching, resting, swimming and erratically swimming were not affected by each treatment but showed increasing trends in the time spent exhibiting these behaviours during multimodal stimuli presentation. The fish used in this study were non-reproductive males with only four individuals (sneaker males) showing high reproductive maturity based on their gonadosomatic index values (GSI). The four reproductive individuals did not differ from non-reproductive in terms of behavioural responses. Experimenting on reproductive males can determine if behavioural responses differ with reproductive status.

Continuing the investigation of behavioural responses to unimodal and multimodal stimuli in laboratory experiments can aid researchers to understand how fishes participate in multimodal communication in their environment (Hebets & Papaj, 2005; Partan & Marler, 2005) and how it can benefit the survival and, in this case, how it may aid in the success of the round goby as an invader. Research in multimodal signalling and synergistic responses in fishes is still a growing field. With similar work done by Kasurak et al. (2012), which presented two sensory modalities to the round goby, my work contributes to this field by using three sensory modalities therefore contributing new data in this field of research.

As previously mentioned, chapter 3 aimed to determine the effects of anthropogenic boat noise on the morphology of the inner ear in black bullhead and determine how regeneration varied with exposure to different sound intensities. Anthropogenic noise is a well-known stressor for fish in their habitats and had shown to negatively impact the behaviour and physiology of fishes (Smith et al., 2004; Wysocki et al., 2006; Purser & Radford, 2011; Purser et al., 2016; Mickle & Higgs, 2018). Chapter 3 results showed that anthropogenic boat noise caused decreases in ciliary bundle counts at 170 and 175 dB re 1 μPa. The fish were able to regenerate ciliary bundles to control levels following noise exposure at 170 dB re 1 μPa but were not able to return to control levels following noise exposure at 175. dB re 1 μPa. I conclude that both sound intensity and exposure time play a significant role in the damage caused by anthropogenic noises in black bullhead. The black bullhead has a broad hearing range and may therefore be more affected by anthropogenic noise sources. If not given the adequate recovery time for regeneration, exposure to high intensity sounds in the wild may cause serious damage that ultimately affect the hearing of this fish and affect its survival. In conclusion, both chapters revealed how the behaviour and physiology of fishes are closely linked to their environment. Continuing to conduct laboratory experiments of this nature can serve to further our understanding on how fishes can perceive their environment and implement strategies to mitigate the spread of an invasive fish species and determine the extent to which anthropogenic noise can affect vulnerable fish species.

Recommendations and future directions

The studies conducted and outlined in my thesis are not without limitations and a few recommendations can made as well as future directions in this area of research. Regarding the second chapter on the round goby, a larger sample size will elucidate certain trends that were seen in the data, which were not significant, and determine the full effects that the stimuli had on quantified behaviours. Increasing the sample size will also allow for randomization of the presentation of stimuli to investigate if this influences behavioural responses exhibited by males and if there are any primer or releaser effects caused by previous exposure to stimuli. I would strongly recommend that future studies should directly compare behavioural responses of non-reproductive males with Type I males (nest guarding) and Type II males (sneaker) (Corkum et al., 1998; Marentette et al., 2009). In chapter 2, only four males were reproductive sneaker males therefore directly testing behavioural responses in all reproductive morphs can aid in clarifying how reproductive status may influence behavioural responses in the round goby (Sisneros & Bass, 2003; Clement et al., 2005; McLennan, 2005; Sisneros, 2009; Kasurak et al., 2012). Since the results in chapter 2 suggest that round gobies are more attracted to multimodal signalling, I would propose that implementing multimodal traps to control the growing population of the round goby in non-native habitats should be considered as it could be a more efficient method to manage this species.

Future studies involving black bullhead and anthropogenic noise exposure experiments should give a longer recovery time following noise exposure to fully determine the time course of ciliary bundle regeneration at higher sound intensities. Additionally, hearing abilities of black bullhead should be assessed during the times that are associated with lower ciliary bundle counts via Auditory Brainstem Response (ABR) to determine if this species exhibits auditory threshold shifts during noise exposure. Studies exposing black bullhead to higher intensities and for longer periods of time should also be conducted (Smith and Monroe, 2016; Pieniazek et al., 2020) as well as using fish at different life stages. I strongly suggest that researchers continue to investigate the effects of anthropogenic noise in black bullhead since the research on this species is somewhat limited and to continue to choose study species that have enhanced hearing ability in future noise experiments. Implementing quiet refuge areas (where anthropogenic sources are prohibited) in areas that are highly trafficked by loud aquatic vessels could provide a safe environment for wild fishes that may be negatively impacted by anthropogenic noise on a daily basis.

The last recommendation I will make is regarding designing field experiments whose results can be compared to the laboratory findings of my thesis. Since the laboratory experiments conducted here simulate an artificial environment, field experiments should also be conducted to observe how the fishes are affected and respond to various types of stimuli, such as natural biological signals or anthropogenic noise sources, within their aquatic environments.

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