The Impact of Anthropogenic Noise on Fish Behavior, Communication, and Development

Julie Butler

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THE IMPACT OF ANTHROPOGENIC NOISE ON FISH BEHAVIOR, COMMUNICATION, AND DEVELOPMENT

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Department of Biological Sciences

by

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B.S. Biology, Texas A&M University, 2013
B.S. Forensic and Investigative Sciences, Texas A&M University, 2013
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<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
</tr>
<tr>
<td>AEP</td>
<td>auditory evoked potential</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AON</td>
<td>anterior octaval nucleus</td>
</tr>
<tr>
<td>BM</td>
<td>body mass</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>CON</td>
<td>caudal octavolateralis nucleus</td>
</tr>
<tr>
<td>CP</td>
<td>central posterior thalamic nucleus</td>
</tr>
<tr>
<td>CRF</td>
<td>corticotropin releasing factor</td>
</tr>
<tr>
<td>CRFBP</td>
<td>CRF binding protein</td>
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<tr>
<td>CRFR1</td>
<td>CRF receptor 1</td>
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<tr>
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<td>CRF receptor 2</td>
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<tr>
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<td>DON</td>
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</tr>
<tr>
<td>Dpf</td>
<td>days post fertilization</td>
</tr>
<tr>
<td>Dpr</td>
<td>days post release</td>
</tr>
<tr>
<td>FFT</td>
<td>fast fourier transformation</td>
</tr>
<tr>
<td>gh1</td>
<td>growth hormone 1</td>
</tr>
<tr>
<td>glul</td>
<td>glutamine synthetase</td>
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</table>
GluR ................................................................. glutamate receptor subunit
GM ........................................................................ gonad mass
GSI ........................................................................... gonadosomatic index
HPA ........................................................................ hypothalamic-pituitary-adrenal
HPI ......................................................................... hypothalamus-pituitary-interrenal
IO ........................................................................ infraorbital canal
ISH ........................................................................ in situ hybridization
LMM ........................................................................ linear mixed model
MABT ........................................................................ malate buffer with tween
MgON ....................................................................... magnocellular octaval nucleus
MD .......................................................................... mandibular canal
MON ......................................................................... medial octavolateralis nucleus
N ............................................................................... naris
OT .......................................................................... otic canal
PFA .......................................................................... paraformaldehyde
PBS .......................................................................... phosphate buffered saline
POMC ..................................................................... pro-opiomelanocortin
PO ........................................................................ postotic canal
PR ........................................................................ preopercular canal
qRT-PCR .............................................................. quantitative reverse transcription polymerase chain reaction
RNA ......................................................................... ribonucleic acid
rpm ........................................................................ revolutions per minute
RT .......................................................................... room temperature
S............................................................... speaker
SL.................................................................... standard length
SO .................................................................... supraorbital canal
SPL .................................................................... sound pressure levels
SSC................................................................. standard sodium citrate
ST..................................................................... supratemporal canal
T........................................................................ trunk canal
TL..................................................................... total length
TON................................................................... tangential octavus nucleus
TS...................................................................... torus semicircularis
TSc................................................................. central TS
TSvl.............................................................. ventrolateral TS
uts1.............................................................. urotensin 1
ABSTRACT

Noise pollution is pervasive to nearly all aquatic and terrestrial ecosystems and was labeled a pollutant of global concern by the World Health Organization in 2011. In the past few decades, underwater ambient noise levels have risen almost 30 dB SPL re: 1 µPa in the frequency range that most fish produce and detect acoustic stimuli due to rises in shipping, oil exploration, and pile driving. Changes to the natural soundscape can impact almost all aspects of an animal’s life. My dissertation research takes an integrative, whole-animal approach to examining how increased background noise impacts fish behavior, physiology, development, and communication. First, I found that social interactions occurring in noisy conditions were less effective. Males spent more time distracted or stressed during territorial fights, resulting in a longer time to fight resolution. Males also changed when and how they courted gravid females. Female hearing capabilities were significantly reduced following noise exposure. Changes to male signal production, female detection capabilities, and possibly the signal itself all interfere with effective social communication. Cumulatively, this resulted in a lower incidence of spawning during noise. Noise exposure also hindered mouthbrooding and maternal care behaviors. Females exposed to noise during brooding were more likely to cannibalize or prematurely release under-developed juveniles. Juveniles that were exposed to noise during development had lower growth rates, higher mortality, and altered social and startle behaviors. Finally, I found that fish possess all components of the proposed inner ear CRF-signaling system and that its expression is mediated by sex, reproductive state, and noise exposure. Because noise-induced changes in expression are dependent on physiological state, it is possible that noise-induced
threshold shifts could also be modulated by reproductive condition. Overall, these results provide one of the most comprehensive whole-animal pictures on how increased background noise impacts fish. By examining subtle, sub-lethal changes to behavior, physiology, and communication, we can better inform conservation efforts before human-influenced noise levels reach potentially lethal levels.
CHAPTER 1. INTRODUCTION

1.1. Fish auditory system

All fishes studied to date are capable of detecting sounds. Unlike tetrapods, fish do not have an external or middle ear, but they do have an inner ear comprised of semicircular canals and three paired otolithic end organs – the lagena, utricle, and saccule (Figure 1.1). The semicircular canals comprise the vestibular system and encode angular accelerations of the head, while the otolithic endorgans are part of the auditory system and encode linear accelerations. Each otolith (calcium carbonate structure) is surrounded by a thin membrane with an underlying sensory macula containing hair cells that are structurally similar to mammalian inner hair cells. Because otoliths are denser than both the fish’s body and surrounding water, they move more slowly in response to a passing sound wave. This lag in otolith movement causes deflection of the hair bundles which, when along the correct axis, opens mechanically-gated ion channels to depolarize the hair cell and cause release of neurotransmitter at the hair cell base to the primary afferent neurons. Action potentials in the afferent neurons are then sent down the auditory nerves (cranial nerve VIII) to the brain.

Sound propagates away from a source as both a pressure wave and particle motion stimulus. Sound pressure waves are due to the alternating bands of compression and rarefaction as the wave propagates underwater; whereas, the particle acceleration component of sound results from the to-and-fro displacement of particles as they transmit vibrations onto neighboring particles. The fish inner ear acts as a biological accelerometer so the particle motion stimulus is thought to be the primary
Figure 1.1. Schematic diagram of the fish inner ear. (A) The fish inner ear consists of three semicircular canals (purple) and three paired end organs (yellow), called otoliths. Each otolith is surrounded by a thin membrane (lilac). (B) The membrane contains a sensory macula region with hair cells oriented towards the otolith. (C) Hair cells contain a single kinocilium (green) and several polarized rows of stereovilli (pink). Information is sent down afferent nerves (cranial nerve VIII) to the brain, while efferent nerves carry information from the brain to the hair cells.

component of sound detected by fishes (De Vries, 1950). However, some fish species possess pressure-transducing morphological specializations that enable them to better detect the pressure components of sound (Sand and Enger, 1973; Tytler and Blaxter, 1977). In clupeids (e.g. herring, menhaden), the auditory bullae, a hollow air-filled bony structure on the part of the skull that encompasses the inner ear, connects with the swim bladder allowing pressure detected by the swim bladder to be transferred to the auditory system (Blaxter et al., 1981). Similarly, Weberian ossicles, small bones that originate from vertebrae, connect the swim bladder and otic capsules in Ostariophysians (superorder containing ~8000 fish species, which includes goldfish and zebrafish) and transduce pressure from the swim bladder to the inner ear similar to how the bones in the middle ear of tetrapods function (Furukawa and Ishii, 1967). In some butterflyfishes (chaetodonids), a laterophysic connection between the preopercular lateral line canal and anterior swim bladder horns allows the mechanosensory and
auditory systems to be sensitive to pressure waves (Webb and Smith, 2000). All fishes have the same basal hearing capability (Radford et al., 2012), but their ability to detect pressure waves varies with the presence or absence of the abovementioned specializations. Some research indicates that the relative swim bladder size and location or the presence of anterior swim bladder extensions that come close to the otic capsules, like those found in cods, cichlids, catfish, and squirrelfishes, can also facilitate detection of sound pressure (Coombs and Popper, 1979; Ladich, 2016; Ramcharitar et al., 2006; Sand and Enger, 1973; Schulz-Mirbach et al., 2012). It is important, therefore, to examine for the presence of pressure-transducing structures and to understand the relationship between the swim bladder and inner ear organs.

The African cichlid fish *Astatotilapia burtoni*, the species I used for my research, does not have a pressure-transducing morphological specialization, but protrusions from the anterior swim bladder compartment extend close to the otic capsule (1-3% of the fish’s body length; Butler et al., 2017). The proximity and location of these protrusions could enhance sound pressure transduction to the inner ear, but this remains untested. My studies refer to sound pressure levels (SPL; dB re: 1 µPa), but future studies should incorporate particle acceleration measurements since *A. burtoni* likely predominately detects the particle displacement components of acoustic stimuli.

Particle displacement is inherently directional, usually taking place along the axis of transmission, but sound pressure is a scalar quantity acting in all directions. Polarized hair bundles in the saccule make it possible for fish to localize a sound source underwater [reviewed in (Popper and Fay, 1993; Sisneros and Rogers, 2016)]. Only deflection of the stereovilli towards the kinocillium will open mechanically-gated ion
channels. The saccule (main hearing organ in most teleost fishes) has patches of directionally-similar hair bundles [reviewed in (Schulz-Mirbach and Ladich, 2016)] to better allow for sound source localization. Similar to how the saccule has areas thought to detect direction along a specific axis, some have suggested that the saccule is also tonotopic (frequency-based spatial organization). In goldfish, lower frequencies are detected by the caudal portion of the saccule and higher frequencies in the rostral portion (Smith et al., 2011). However, toadfish have no tonotopic organization to their saccule. Instead, it is proposed that micromechanical mechanisms are used for frequency discrimination. Although sound source localization and frequency discrimination is still not well understood in fishes, it is well accepted that disruption of a fish's ability to localize a sender could have detrimental effects on reproductive success.

1.2. Mehanosensory lateral line system

The mehanosensory lateral line system exists in all fishes and some aquatic and semi-terrestrial amphibians, and detects water movements occurring close to the body surface of the animal (Coombs, 1994; Coombs et al., 1996; Dijkgraaf, 1963; McHenry and Liao, 2014). The functional units, called neuromasts, are composed of support cells and sensory hair cells (Figure 1.2A). Each hair cell has a single kinocillium and several polarized rows of stereovilli which project into a gelatinous cupula (Dijkgraaf, 1963). In fishes, neuromasts are either located on the skin surface of the fish (superficial neuromasts) or embedded in bony canals of the dermis [canal neuromasts; Figure 1.2B; (Webb, 1989)]. Because of the difference in their morphology and location, canal and superficial neuromasts encode different stimulus properties (Chagnaud and
Superficial neuromasts primarily detect velocity, while canal neuromasts are sensitive to the acceleration differences between the fish and surrounding water. Water movements near the surface of the fish create viscous drag which deflects the cupula and opens mechanotransduction channels in the stereovilli to depolarize the sensory cells. This depolarization causes the release of neurotransmitter at the base of the hair cells to modulate the spontaneous discharge patterns of the primary afferent neurons. These action potentials are then sent down lateral line nerves, where they, like the auditory nerves, enter the hindbrain. From there, mechanosensory information is sent to higher processing centers of the brain where it is integrated with other sensory information to ultimately influence complex behavioral decisions (Butler and Maruska, 2016a).

**Figure 1.2.** Neuromast structure and distribution of the mechanosensory lateral line system in the African cichlid fish, *Astatotilapia burtoni*. (A) Lateral line neuromasts are composed of support cells (not shown) and sensory hair cells, each of which contains a single kinocilium and several rows of stereovilli, projecting up into a gelatinous cupula. Afferent nerves deliver mechanosensory information from water movements produced near the fish and transmit it to the brain. (B) The *A. burtoni* lateral line system consists of seven cranial canals and a disjunct trunk canal. Canal neuromasts (large black ovals) lie inside bony canals (grey shading) embedded within the dermis and each neuromast is located between adjacent canal pores (open ovals). Superficial neuromasts (small black circles) are located on the skin surface around the naris, in rows or clusters around canals, and in two rows down the length of the caudal fin. IO, infraorbital canal; MD, mandibular canal; N, naris; OT, otic canal; PR, preopercular canal; PO, postotic canal; SO, supraorbital canal; ST, supratemporal canal; T, trunk canal. From Butler and Maruska, 2015.
1.3. **Mechanosensory versus auditory communication**

At one point in history the lateral line system was thought to be an accessory hearing organ (van Bergeijk, 1964). Today, electrophysiological, morphological, neuroanatomical, and behavioral evidence indicates that while both the lateral line and auditory inner ear can be stimulated by the same source, they are in fact two distinct systems [reviewed in (Braun and Sand, 2014; Kalmijn, 1988; Kalmijn, 1989)]. Importantly, acoustic stimuli can be detected by both the auditory and mechanosensory systems, but this is dependent on frequency and distance, such that perception of low frequencies at close range is a multimodal response. Due to enhanced sound propagation underwater, particularly at low frequencies, acoustic communication can occur across relatively large geographical spaces, but the lateral line system only detects close-range (usually within ~1-2 body lengths) stimuli. Anthropogenic noise can potentially impact both hair cell-based sensory systems, so it is important to investigate their relative role in social communication to better understand how disruption of these sensory channels might impact reproduction and territorial interactions.

1.4. **Production of auditory and hydrodynamic signals**

Over 800 species of fishes are known to produce sounds. Soniferous fishes are phylogenetically widespread and the sound type and production mechanism is equally diverse [reviewed in (Fine and Parmentier, 2015)]. The primary methods of sound production include the use of the swim bladder or rubbing together of skeletal elements (stridulation). Many sound-producing fishes, such as toadfishes and drums, have sonic muscles that are attached to their swim bladder (Fine and Parmentier, 2015; Ladich and
Fine, 2006; Lobel et al., 2010). Rapid contractions of the sonic muscles cause the swim bladder to contract and expand at a rapid rate which produces a drumming or humming sound (Demski et al., 1973; Ladich and Fine, 2006). The anatomical connections between the sonic muscles and swim bladder can affect the type of sound produced. Stridulation occurs from the rubbing together of skeletal parts or teeth, such as pharyngeal jaws (Colleye et al., 2013; Parmentier et al., 2013). Typically, sounds produced via swim bladder mechanisms dominate at lower frequencies (< 1 kHz), but stridulation-related sounds can extend up to 4 kHz (Zeyl et al., 2016).

The swim bladder may still be responsible for sound production even when sonic muscles are absent. For example, in the Nile tilapia Oreochromis niloticus, sound production is due to the combined backwards movement of the pelvic and pectoral girdles and forward movements of the anal fin (Longrie et al., 2009). This causes contractions of the musculature that compresses the ribcage and swim bladder to produce a sound. In Astatotilapia burtoni, sound production is associated with a body quiver (Figure 1.3A-C), which involves similar movements to those associated with sound production in tilapia (Maruska et al., 2012). In addition, swim bladder morphology is similar between the two species (Butler et al., 2017), suggesting a similar sound production mechanism in A. burtoni, but this remains untested.

Any movement underwater will inevitably generate hydrodynamic stimuli that can be detected by the lateral line system of nearby fish. Fish employ many social behaviors that involve fin and body motions [Figure 1.3D-I; (Aronson, 1949; Barlow, 2002; Dijkgraaf, 1963; Enger et al., 1989; Fernald, 1977; Fernald and Hirata, 1977; Mackereth and Keenleyside, 1993; Munro and Pitcher, 1985; Noble and Curtis, 1939; Thresher,
Astatotilapia burtoni produce auditory and hydrodynamic signals during social interactions. (A) Male A. burtoni produce facultative courtship sounds associated with reproductive body quivers. (B-C) Representative waveform (B) and spectrogram (C) of a male courtship sound. (D-H) Dominant male aggressive behaviors (e.g. lateral display, (D); border fight, (E); frontal threat (F) and reproductive behaviors (e.g. quiver, (G); lead/tail waggle, (H) produce hydrodynamic signals. (I) In some cichlids, parents call their young back to the buccal cavity using tail and fin movements. Modified from Maruska and Fernald 2010; Maruska et al., 2012; Butler and Maruska, 2016b.

1984), and these have even been called ‘signal movements’ by early neuroethologists. For example, an aggressive lateral display involves one fish orienting parallel or perpendicular to his opponent, fully erecting its dorsal, anal, and caudal fins, and distending its jaw to create a visual display of larger size. Many fishes accompany this by gently to vigorously shaking their body. This behavior, and many other common aggressive and reproductive behaviors, generate water movements that can be detected by conspecifics. Body and tail movements generate hydrodynamic flow fields consisting of low-frequency stimuli (< 10 Hz) coupled with higher frequency acceleration.
components (Bleckmann et al., 1991) indicating that these stimuli can stimulate both superficial and canal neuromasts of the lateral line system.

1.5. Role of acoustic and hydrodynamic information in fish social interactions

Acoustic communication can provide vital information about the sender’s species, sex, reproductive or social state, and motivation. Over 800 species of fish are known to produce sounds, most of which occur during reproduction. These close-range acoustic signals used during courtship and mate choice can provide females with information on male size and condition. Most courtship sounds dominate at low frequencies and are intended for relatively close-range communication, making them susceptible to acoustic masking from increased background noise. Darters, gobies, and sculpins typically produce pulsed sounds under 200 Hz that are almost always associated with agonistic or reproductive interactions and only function over a distance of a few centimeters (Lugli et al., 2003; Zeyl et al., 2016). Toadfish and midshipman males establish nests in shallow intertidal zones and form choruses to attract gravid females. Although their sounds are relatively loud [125 SPL dB re: 1 µPa; (Barimo and Fine, 1998)], the shallow water means females only respond to sounds produced within 5-8 m (Fine and Lenhardt, 1983). The ability to recruit reproductively receptive females to a spawning territory is extremely important for site-attached animals living in noisy acoustic environments.

Territorial *A. burtoni* males produce facultative courtship sounds during reproductive body quivers (300-700 Hz peak frequency) performed at reproductively receptive females, and gravid females prefer males associated with courtship sounds
(Maruska et al., 2012). Larger males produce more courtship sounds, and body size correlates with mean peak frequency of the sound, indicating that acoustic signals are an honest indicator of male quality. Even if increased background noise does not completely mask acoustic communication, if it disrupts the female’s ability to distinguish temporal aspects of the call, it could have implications for mate preference and sexual selection.

Mechanosensory information is also used for a variety of fish behaviors. The role of water movement cues in mediating obstacle avoidance (Baker and Montgomery, 1999; Kulpa et al., 2015; Montgomery et al., 1997; Windsor, 2014), rheotaxis [orientation within a current; (Montgomery et al., 1997)], schooling (Pitcher et al., 1976), and predatory-prey interactions (Coombs and Patton, 2009; Schwalbe et al., 2012; Schwalbe et al., 2016) has been well-studied, but many fish social behaviors produce water movements that can be detected by the lateral line system of nearby fish. In most aggressive encounters, fish typically begin with non-contact aggressive behaviors but escalate to costlier contact behaviors (Enquist et al., 1990; Leiser et al., 2004). In A. burtoni, males use mechanosensory cues for mutual assessment when deciding whether to engage in a territorial dispute or to retreat (Butler and Maruska, 2015). In addition, they predominately use non-contact aggressive behaviors during territorial fights, and when mechanoreception is disrupted, they rely on more dangerous contact behaviors. Research in other fishes suggests that mechanosensory or vibrational communication is used to attract potential mates and synchronize spawning (Marchesan et al., 2000; Medina et al., 2013; Satou et al., 1991; Satou et al., 1994), but this has not been tested yet in A. burtoni. Because any movement underwater will
produce a hydrodynamic cue, understanding the role of hydrodynamic signaling during social interactions is important. In addition, any disruption of mechanosensory communication (e.g. from increased background noise or heavy metal runoff) can have detrimental effects on fish social communication and ultimately reproductive success and species persistence.

1.6. Central processing of auditory and mechanosensory stimuli

Auditory and mechanosensory information arrive separately at the hindbrain via branches of the eighth (VIII) nerve and lateral line nerves (anterior and posterior), respectively (Edds-Walton, 1998; Highstein et al., 1992). Input from the saccule, lagena, and utricle enters the hindbrain octavolateralis column. The descending octaval nucleus (DON) is generally accepted to be the primarily auditory processing region (Edds-Walton and Fay, 1998; Edds-Walton, 1998), although the anterior, tangential, and magnocellular octaval nuclei (AON, TON, MgON, respectively) also receive auditory input, and all four nuclei receive some vestibular information (Echteler, 1985; McCormick and Braford Jr, 1993; McCormick and Braford Jr, 1994; McCormick and Wallace, 2012). Like the auditory system, mechanosensory information arrives at the hindbrain octavolateralis column (Maruska and Tricas, 2009; McCormick, 1983; McCormick, 1989; Tomchik and Lu, 2005; Wullimann and Grothe, 2014). Afferent fibers from superficial and canal neuromasts arrive separately at the brain, and terminate primarily in the medial octavolateralis nucleus (MON) with some terminating in the caudal octavolateralis nucleus [CON; (McCormick, 1983; Meredith, 1984; Wullimann and Grothe, 2014)]. From the hindbrain, ascending fibers primarily project to the
midbrain torus semicircularis (TS) where auditory and mechanosensory information is partially integrated (Echteler, 1984; Luiten, 1975; McCormick, 1982; McCormick, 1989). The central nucleus (TSc) typically responds to auditory stimuli whereas the ventrolateral nucleus (TSvl) responds to hydrodynamic stimuli, with some neurons responding to both (Edds-Walton, 2016; Fay, 2001). Information then gets routed through the central posterior thalamic nucleus [CP; (Finger and Tong, 1984)] and sent to various hypothalamic and telencephalic structures, including those involved in mediating complex social behaviors (Butler and Maruska, 2016a; Finger and Tong, 1984; Murakami et al., 1986; Wullimann and Grothe, 2014).

1.7. Hypothalamic-Pituitary-Interrenal (HPI; stress) axis of fishes

The mammalian hypothalamic-pituitary-adrenal (HPA) axis is a neuroendocrine system that helps regulate many homeostatic systems of the body including digestion and energy balance [reviewed in (Dallman and Bhatnagar, 2010; Nieuwenhuizen and Rutters, 2008)]. It integrates internal and external factors, like illness, temperature, and social stress, to allow organisms to effectively adapt to their environment and ultimately increase survival. Fish do not have an adrenal gland, but instead, glucocorticoids are produced by the paired interrenal glands located by the head kidney (Donaldson, 1981; Ilan and Yaron, 1980; Morandini et al., 2014). In the HPA/I axis, corticotropin releasing factor (CRF) is produced in the hypothalamus and released into the adenohypophysis (i.e. anterior pituitary) where it binds CRF receptors to stimulate the production of proopiomelanocortin (POMC). Cleavage products of POMC, one of which is adrenocorticotropin hormone (ACTH), are released into the bloodstream, and when
ACTH binds melanocortin 2 receptors in the adrenals/interrenals, it stimulates production of glucocorticoids (e.g. cortisol in teleost fishes). Many other neuropeptides and neurochemicals, like arginine vasopressin (or vasotocin in fishes) and serotonin can also act in concert with CRF to regulate the stress axis (Fuller, 1992; Herman and Cullinan, 1997; Pariante and Lightman, 2008).

Stress has negative impacts on reproductive behavior and physiology across taxa (Sapolsky et al., 2000). Chronic stress can inhibit the reproductive axis at the level of the brain, pituitary, and gonads [reviewed in (Rivier and Rivest, 1991)]. CRF can act as a neuromodulator in the brain to decrease gonadotropin-releasing hormone (GnRH) release (Nikolarakis et al., 1986) and affect luteinizing hormone (LH) pulsatile release from the pituitary (Petraglia et al., 1987). Stress can also decrease the stimulatory effect of gonadotropins on sex steroid secretion by the gonads (Fabbri et al., 1990). Although the association between stress and altered reproductive measures has been well-studied, the link between noise exposure, stress, and reproductive fitness in fishes remains unexplored.

1.8. Underwater anthropogenic noise

Underwater anthropogenic noise has risen rapidly over the past several decades. Due to increases in shipping, pile driving, oil exploration, and other human activities, ambient underwater noise levels have increased ~30 SPL dB re: 1 µPa over the 0.1-2 kHz range (Board, 2005; Purser and Radford, 2011), which coincides with the frequencies at which fishes hear best and produce sounds (Figure 1.4). While the bandwidth of most anthropogenic sounds extends up to 10 kHz, the dominant frequency
Figure 1.4. Relative amplitude of noise playback (used in Chapters 2, 3, and 4; ‘Noise file’, red line), boat noise (blue line), *Astatotilapia burtoni* male courtship sound (‘Male sound’, grey line), and ambient tank (green line) sound levels. The created noise file, motor boat recording, and courtship sound were played through an underwater speaker and the relative amplitude in the aquarium was recorded. In addition, the amplitude when no sound was being played was determined. While boat noise has peak frequency components < 200 Hz, the noise file contains frequency components encompassing the entire range of *A. burtoni* hearing.

Components are often < 1000 Hz, which is in the hearing range of most fishes (Table 1.1). High intensity sounds (e.g. pile driving, airguns, sonar) are often short in duration (< 1 sec) while lower intensity sounds (i.e. shipping, drilling) last for longer periods of time and, in some environments, can even be continuous (McGregor et al., 2013). Because of the impulsive nature of high intensity sounds, they have been studied more readily than low intensity sounds, and most of the research examining the impact of underwater anthropogenic noise has focused on marine mammals or non-vocalizing fish species [reviewed in (Popper and Hastings, 2009a; Popper, 2003; Popper and
More research is needed in social fishes that use acoustic communication to fully understand how fish might cope with noise pollution.

Table 1.1. List of underwater anthropogenic sounds and their characteristics. Modified from (Hildebrand, 2009; McGregor et al., 2013)

<table>
<thead>
<tr>
<th>Sound Source</th>
<th>Sound Level (dB re: 1 μPA)</th>
<th>Bandwidth (Hz)</th>
<th>Major Frequency (Hz)</th>
<th>Duration (ms)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pile driving</td>
<td>243-257</td>
<td>20-100,000</td>
<td>100-500</td>
<td>0.05</td>
<td>Continuous</td>
</tr>
<tr>
<td>Airgun array</td>
<td>260-262</td>
<td>50-100,000</td>
<td>10-120</td>
<td>0.03-0.06</td>
<td>Continuous</td>
</tr>
<tr>
<td>Military sonar</td>
<td>214-240</td>
<td>100-8,000</td>
<td>Various</td>
<td>0.5-100</td>
<td>Continuous</td>
</tr>
<tr>
<td>Large shipping vessel</td>
<td>180-190</td>
<td>5-30,000</td>
<td>&lt;200</td>
<td>Continuous</td>
<td>Continuous</td>
</tr>
<tr>
<td>Small boats</td>
<td>160-180</td>
<td>20-10,000</td>
<td>&lt;1000</td>
<td>Continuous</td>
<td>Continuous</td>
</tr>
<tr>
<td>Dredger</td>
<td>168-186</td>
<td>10-10,000</td>
<td>100-500</td>
<td>Continuous</td>
<td>Continuous</td>
</tr>
<tr>
<td>Wind turbine</td>
<td>142-151</td>
<td>16-20,000</td>
<td>30-200</td>
<td>Continuous</td>
<td>Continuous</td>
</tr>
<tr>
<td>Drilling</td>
<td>145-190</td>
<td>10-10,000</td>
<td>&lt;100</td>
<td>Continuous</td>
<td>Continuous</td>
</tr>
<tr>
<td>Acoustic deterrent devices</td>
<td>132-200</td>
<td>2,000-30,000</td>
<td>Various</td>
<td>0.015-0.6</td>
<td>Continuous</td>
</tr>
</tbody>
</table>

Anthropogenic noise has the potential to influence animals on multiple levels of biological organization and on a larger population level [Figure 1.5; (Board, 2005)]. Exposure to anthropogenic sounds can simultaneously impact fish endocrine systems, neural systems, reproductive organs, and many other vital physiological systems. These changes can ultimately affect 'life functions' that lead to changes in maturation and reproductive rates. Finally, changes on this scale can also impact populations and ultimately species survival. By testing for noise-induced effects at multiple levels, my research aims to understand how anthropogenic sounds ultimately affect fish reproduction in a soniferous, territorial fish species.
Figure 1.5. Population consequence model of acoustic disturbance. By testing for noise-induced effects at multiple levels (indicated by dashed box), I provide a more complete picture of how noise exposure impacts a territorial, soniferous fish species. Modified from NRC, 2005.

1.9. Model system: *Astatotilapia burtoni*

The African cichlid fish *Astatotilapia burtoni*, an emerging model system for neuroethology research, is a highly social fish with well-characterized aggressive and courtship behaviors (Fernald, 1977; Fernald and Hirata, 1977). Male *A. burtoni* live in territorial systems and exist as two distinct phenotypes (dominant and subordinate; Figure 1.6), which they can rapidly and reversibly switch between depending on their social environment, and changes in social status are accompanied by a suite of behavioral, physiological, and morphological changes (Maruska, 2014; Maruska and Fernald, 2014a). Dominant, territorial males are brightly colored, aggressively defend their spawning territory from other males, and actively court reproductively receptive females. Subordinate, non-territorial males are drably colored, physically resemble females, and are reproductively suppressed. Social status is tied to reproductive success, feeding opportunities, and growth rate (Hofmann et al., 1999; Maruska and Fernald, 2014a), and in *A. burtoni* is dependent on the male’s ability to successfully defend his territory. To do this, dominant males use a variety of aggressive behaviors, such as lateral displays, border fights, frontal threats, chases, and biting. During many
of these behaviors, fish are in close proximity to each other, but not physically touching. The fin and body movements used during these behaviors create visual displays, but they also produce acoustic stimuli that can be detected by the mechanosensory and auditory systems of nearby fish (Butler and Maruska, 2015; Butler and Maruska, 2016b).

Figure 1.6. Schematic drawings of the African cichlid fish *Astatotilapia burtoni*. Dominant males (A) are brightly colored whereas subordinate males (B) are drably colored and physically resemble females. Gravid females (C) are characterized by a distended abdomen due to large, ready-to-spawn eggs. Brooding females (D) have a distended buccal cavity to hold the developing fry.

Dominant males actively court females using a variety of behaviors including body quivers, tail waggles, and leads. During a body quiver, a dominant male displays his anal fin to a gravid female while vigorously shaking his body. This is often followed by exaggerated waggles of the tail while leading the female back to the spawning territory. Like aggressive behaviors, their courtship behaviors also produce hydrodynamic cues that females can potentially use for mate choice. During spawning, the male and female perform many circling bouts during which the male gently prods eggs from the female, the female takes up the eggs into her buccal cavity, and then nips at the eggspots on the male’s anal fin while he releases sperm [Figure 1.7; (Salzburger et al., 2007)]. The female then carries these fertilized eggs in her mouth until the fry are fully developed (~2 weeks). This extreme form of parental care, called mouthbrooding, causes forced starvation of the female and results in many other behavioral and
physiological consequences. Upon developmental maturity of her brood, the female will release her fry and continue to provide parental care for ~2 days during which time she will take them back into her buccal cavity when threatened. After mouthbrooding, females have a ~26-day recovery period during which time vitellogenesis occurs to prepare females for another spawning event. The well-characterized courtship and reproductive behaviors make *A. burtoni* an excellent model system to test for noise-induced effects on reproductive success.

Figure 1.7. Female *Astatotilapia burtoni* take up eggs into her buccal cavity during spawning. Circling bouts during spawning start with a male gently prodding females to facilitate egg release. The female then takes the eggs into her mouth and nips at the eggspots on the male’s anal fin while he releases sperm to fertilize the eggs in her buccal cavity. Modified from Salzburger et al., 2007.

Molecular techniques, such as quantitative qPCR and *in situ* hybridization, are relatively easy to perform with *A. burtoni* since they have a fully sequenced and annotated genome (Brawand et al., 2014). Because they have been used as a model system for neuroethology research for > 30 years, there is a plethora of neuroendocrine data on hormonal and neuropeptide changes associated with social status and reproductive state changes. This, in addition to their territoriality and use of acoustic communication, makes them an ideal model system to investigate the reproductive consequences of life in a noisy environment.
1.10. Specific Aims

The overall goal of my dissertation is to investigate how exposure to anthropogenic noise impacts fish behavior, physiology, communication, and development. While past studies have investigated the impact of noise on each of these components individually in other species, no study has taken an integrative approach to look at how noise impacts fish across multiple levels of biological organization. The following specific research topics will be addressed:

1.10.1. Chapter 2. Impact of anthropogenic noise on behaviors and social communication

In chapter 2, I test multiple hypotheses relating to the impact of noise on social behaviors and communication. First, I examined the impact of noise on territorial and reproductive interactions. By comparing territorial interactions occurring in noisy or control environments, I show that a noisy environment affects the structure of agonistic interactions and ultimately leads to a longer time to fight resolution. During noise, males also increased their use of visual signaling by displaying their eyebar for greater periods of time. In reproductive interactions, males do not change the number of reproductive behaviors, but they change where and how they perform these behaviors. These changes in signal production decrease the likelihood of female detection. Ultimately, females are less responsive to male courtship attempts and have lower incidence of spawning when noise was present.

I also test the hypothesis that exposure to noise negatively impacts hearing capabilities. My results show that gravid females, the primary intended receivers of acoustic communication in A. burtoni, have decreased sensitivity at 200 and 300 Hz.
when compared with control gravid females. This frequency range represents a major component of male courtship sounds, and shows the greatest change in reproductive-state plasticity in hearing sensitivity between gravid and non-reproductive females (Maruska et al., 2012). Coupled with changes to signal production by males and potential masking of acoustic signals, these data indicate that anthropogenic noise affects all three components of social communication: signal production, signal detection, and the signal itself. This chapter is formatted for submission to Animal Behaviour and is partially redundant with Chapter 1.

1.10.2. Chapter 3. Impact of anthropogenic noise on maternal care behaviors and juvenile development

In chapter 3, I investigate the impact of anthropogenic noise on mouthbrooding. I found that mouthbrooding females exposed to noise have decreased brooding success. This is due to an increase in brood cannibalization or release of under-developed juveniles. However, there are still many questions regarding the mechanisms responsible for these changes. Also in chapter 3, I test the hypothesis that exposure to noise will negatively impact juvenile development. I found that a single exposure to noise during a critical developmental stage results in lower growth rates and higher mortality. Juveniles exposed to noise during development also had altered shoaling and startle behaviors and a delayed onset of adult typical social behaviors and coloration. This is one of the first studies to demonstrate long-term effects from a single exposure to anthropogenic noise during development. These data also provide important information for future studies looking at the mechanisms underlying observed changes to maternal care behaviors and juvenile development.
1.10.3. Chapter 4. Do fish possess an inner ear CRF-signaling system that may function to protect against noise-induced hearing loss?

In chapter 4, I measure expression levels of the CRF signaling system, glutamine synthetase, and growth hormone in saccules from control and noise-exposed animals of varying reproductive state. Components of the CRF signaling system were expressed in a sex and reproductive state-dependent manner. After noise exposure, crf ligand and crfr2 expression were increased, but crfr1 expression decreased. Based on what is known about CRF receptor activation in the mammalian inner ear, lower CRF-R1 signaling and higher CRF-R2 signaling could work together to allow the inner ear to attenuate prolonged noise exposure. This study lays the ground work for functional manipulation studies with CRF agonists and receptor antagonists to test if the CRF signaling system has a conserved function to protect against noise-induced hearing loss. In addition, I found that noise exposure resulted in damage to sensory hair cells. In other fishes, growth hormone is involved in cell proliferation and hair cell regeneration. Interestingly, gh1, the gene encoding growth hormone, is the only measured gene that did not vary with sex, reproductive state, or noise exposure. However, this could be because samples were collected immediately after noise-exposure and are before the rise in growth hormone observed in other species.

1.11. References


CHAPTER 2. ANTHROPOGENIC NOISE IMPAIRS SOCIAL COMMUNICATION DURING AGGRESSIVE AND REPRODUCTIVE ENCOUNTERS

2.1. Introduction

Communication is a vital aspect of all social interactions. Animals rely on signals encoding information about the sender’s species, sex, motivation, reproductive state, and identity. Communication, as defined by (Bradbury and Vehrencamp, 1998), involves a “sender producing a signal that conveys information and a receiver making a decision on how to respond to that signal”. For communication to be effective, the signal itself, the receiver’s sensory physiology, and the receiver’s response must be in tune with the environmental conditions that carry the signal (Cole, 2013). Disruption of this communication can have detrimental impacts on both the sender and receiver.

Unfortunately, anthropogenic (human-made) noise has now become a pervasive pollutant to almost all aquatic and terrestrial environments (Halfwerk and Slabbekoorn, 2015). Shipping travel, sonar use, and oil exploration all contributed to the rise in ambient underwater sound levels, which have increased over 30 SPL dB re: 1 µPa in the frequency range that most fish produce and detect acoustic stimuli (Board, 2005; Crovo et al., 2015; Purser and Radford, 2011; Radford et al., 2014; Scholik and Yan, 2001; Scholik and Yan, 2002a; Scholik and Yan, 2002b; Vasconcelos et al., 2007).

Anthropogenic noise is linked to changes in hearing capabilities (Casper et al., 2013), schooling and shoaling behaviors (Herbert-Read et al., 2017), development (Davidson et al., 2009; Nedelec et al., 2015), learning and memory (Ferrari et al., 2018), stress physiology (Anderson et al., 2011; Crovo et al., 2015), foraging (Bracciali et al., 2012; McLaughlin and Kunc, 2015), predator avoidance (Chan et al., 2010), and social
behaviors (Algera et al., 2017; Bruintjes and Radford, 2013; Bruintjes and Radford, 2014; de Jong et al., 2018a; Sebastianutto et al., 2011) in diverse fish species. However, there remains a paucity of research on how anthropogenic noise impacts social communication.

Over 800 species of phylogenetically diverse fishes are known to produce sounds, mainly during reproduction (Fine and Parmentier, 2015). Acoustic signals are typically produced by males during courtship and can provide females with information on male size and condition for use during mate choice. Most courtship sounds have dominant energy at low frequencies and are intended for relatively close-range communication, making them susceptible to acoustic masking from increased background noise. For example, darters, gobies, and sculpins typically produce pulsed sounds under 200 Hz that are almost always associated with agonistic or reproductive interactions and only function over a distance of a few centimeters (Lugli et al., 2003; Zeyl et al., 2016). Toadfish and midshipman males establish nests in shallow intertidal zones and form choruses to attract gravid females. Although their sounds are relatively loud [125 SPL dB re: 1 µPa; (Barimo and Fine, 1998)], the attenuation in shallow water means females only respond to sounds produced within 5-8 m (Fine and Lenhardt, 1983). The ability to recruit reproductively receptive females to a spawning territory is extremely important for site-attached animals living in noisy acoustic environments.

Any movement underwater will inevitably generate hydrodynamic stimuli that can be detected by the lateral line system of nearby fish. Fish employ many social behaviors that involve fin and body motions (Butler and Maruska, 2016b), referred as ‘signal movements’ by early neuroethologists. For example, an aggressive lateral display
involves one fish orienting parallel or perpendicular to his opponent, fully erecting its dorsal, anal, and caudal fins, and distending its jaw to create a visual display of larger size. During this visual display, many fishes also gently to vigorously shake their body. This behavior, and many other common aggressive and reproductive behaviors, generate water movements that can be detected by conspecifics. Body and tail movements generate hydrodynamic flow fields consisting of low-frequency stimuli (< 10 Hz) coupled with higher frequency acceleration components (Bleckmann et al., 1991) indicating that these stimuli can stimulate both superficial and canal neuromasts of the lateral line system. Any disruption of mechanosensory communication can have detrimental effects on fish social communication and ultimately reproductive success and species persistence.

Boat noise is one of the most prominent sources of anthropogenic noise. For animals living in shipping lanes or harbors, the noise from motors can be near continuous. While the peak frequency of boat noise varies with vessel size, motor output, and speed, it is typically below 1000 Hz, which corresponds to peak hearing range for many fishes (McCormick et al., 2018). Playback of boat noise or white noise affects hearing capabilities and can result in a physiological stress response in fishes (Casper et al., 2013; Crovo et al., 2015). Anthropogenic noise also affects territorial behaviors in gobies (Sebastianutto et al., 2011), nest maintenance and defense behaviors in cichlids (Bruinjes and Radford, 2013), and social communication and spawning success of gobies (de Jong et al., 2018a). While studies have examined the impact of noise on behavior, signal production, or sensory capabilities individually, no study has tested for noise induced-impacts on social behaviors and communication as a
whole. Anthropogenic noise has the potential to impact social behaviors, signal production, signal transmission, and the receiver’s physiology and behavioral response. By examining noise-induced impacts on multiple components of social communication, we identify subtle changes that can have major consequences for predator avoidance and reproductive success. This type of subtle noise-induced changes to behavior and communication can serve as an early indicator of potentially harmful impacts of anthropogenic noise on fish. Subtle changes, as opposed to major organ damage or even death, are possibly more important for conservation efforts.

The African cichlid fish *Astatotilapia burtoni* is an excellent system to investigate the impacts of underwater noise on social interactions. Their social behaviors and communication are well-documented and described (Fernald and Hirata, 1977; Maruska and Fernald, 2010b; Maruska and Fernald, 2018). Male *A. burtoni* live in a territorial system as two main phenotypes on a continuum: dominant/territorial and subordinate/non-territorial. They are able to rapidly and reversibly switch between phenotypes depending on their social environments (Maruska and Fernald, 2011; Maruska and Fernald, 2013). Dominant males actively defend their spawning territory from other males using a variety of agonistic behaviors, such as chases, bites, lateral displays, and frontal threats (Fernald and Hirata, 1977). While there is no evidence for intentional sound production during agonistic interactions, these behaviors do produce hydrodynamic stimuli, and detection of these water-movements are essential for mutual assessment and fight escalation (Butler and Maruska, 2015). During courtship, males use visual, acoustic (both auditory and hydrodynamic), and chemical signals to entice females to their territories for spawning (Maruska and Fernald, 2012; Maruska et al.,
Dominant males actively court females using body quivers, tail waggles, and leads. During a body quiver, a male displays his anal fin to a gravid female while vigorously shaking his body. This is often followed by exaggerated waggles of the tail while leading the female back to the spawning territory. Like aggressive behaviors, their courtship behaviors produce water movements that females can potentially use for mate choice. Dominant *A. burtoni* males also produce facultative courtship sounds during reproductive body quivers (300-700 Hz peak frequency), and gravid females prefer males associated with courtship sounds (Maruska et al., 2012). Larger males produce more courtship quivers with sounds, and body size correlates with mean peak frequency of the sound, indicating that acoustic signals are an honest indicator of male quality. Because *A. burtoni* rely on acoustic communication during social interactions, it is possible that underwater noise could interfere with this communication and therefore alter social behaviors.

Here, we examined the impact of underwater noise on territorial male-male interactions and reproductive male-female interactions, both of which are necessary for species persistence. In both contexts, fish were less likely to interact with each other in a noisy environment compared to silence. When they did interact, fish performed the same number of behaviors, but how they used them (i.e. sequence, timing) differed between the sound conditions. Finally, females had reduced hearing capabilities, were less responsive to male courtship attempts, and had a lower incidence of spawning during noise exposure. Overall, these data indicate that anthropogenic noise has negative impacts on social behaviors, with changes to signal production and ultimately decreased social communication. Disruption of social communication during these vital
behaviors likely has negative impacts on predation rates, reproductive fitness, and species persistence.

2.2. Materials and methods

2.2.1. Experimental animals

Laboratory bred *Astatotilapia burtoni* were maintained in community aquaria at conditions simulating their natural environments (pH = 7.6-8.0; 28-30 °C; 12 L:12 D diurnal cycle). Adults were fed cichlid flakes daily and brine shrimp twice weekly. All community aquaria contained 2-3 partial terracotta pots to serve as spawning territories. A total of 56 individuals were used (standard length = 44.750 ± 6.181 mm; body mass: 2.503 ± 0.826 g). All experiments were performed in accordance with the recommendations and guidelines stated in the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, 2011. All animal care and collection procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Louisiana State University, Baton Rouge, LA.

2.2.2. Sound exposure protocol

All behavior experiments occurred in 38 L glass aquaria placed on several layers of foam insulation to isolate them from outside vibrations. Each tank (49.5 cm L x 25.4 cm W x 29.2 cm H) was divided into two compartments by an opaque acrylic divider (front compartment: 35 cm L; back: 14.5 cm L). The back compartment contained an underwater speaker (UW-30, frequency response 100 Hz – 10 kHz). The speaker was placed in a separate compartment from the behavior trials because males used the
inside of the speaker or area immediately behind the speaker as their territory when sound was not on. The speaker was suspended from a PVC frame above the tank so that no part of the speaker touched the tank. All behavior experiments occurred in the front compartment.

To create a “noisy” environment, a sound file was created in Audacity v2.1.1 (http://audacityteam.org/) comprised of random pure tones ranging from 100-2000 Hz [the hearing range of *A. burtoni* (Maruska et al., 2012)]. Tone order and duration (0.5-4.0 sec) were randomized. Each sound file was approximately 5 min but looped for the duration of the 30 min behavior trial. Sound files were amplified (TOA, CA-160) before being played through the submerged underwater speaker. The amplifier was adjusted so that the sound level was ~140 SPL dB re: 1µPa immediately above the territory (Figure 2.1). During control trials, all equipment was present, but no sound file was played through the speaker.

We chose to use pure tones within the hearing range of *A. burtoni* (Maruska et al., 2012) instead of boat playback or broadband noise for several reasons. The abovementioned sound file was easier to characterize and reproduce within aquaria. Sound playback in small aquaria cannot adequately mimic natural sound conditions, even under ideal conditions (Akamatsu et al., 2002). In addition, this allowed us to examine in related studies not described here whether or not there was a frequency-dependent impact on behaviors. While the sound stimulus used in this study does not represent a natural stimulus (i.e. playback of motorized boat), it has similar characteristics including predominantly low frequencies and random nature (Figure 1.4).
Figure 2.1. Schematic diagram of experimental tank with sound pressure levels (SPL) for each quadrant. Sound varies with proximity to the speaker but not with depth. White circles represent the bottom third of the tank (just above the gravel), grey represents mid-water column, and black circles represent the top third of the water column. The experimental tank is a 38 L glass aquarium (49.5 cm L x 25.4 cm W x 29.2 cm H). Brown semicircle represents the halved-terra cotta pot used as a spawning shelter by males.

2.2.3. Aggressive behavior protocol

To examine the impact of underwater noise on territorial interactions, we induced aggressive interactions between two males occurring in either silent ($N = 7$ trials) or noisy ($N = 9$ trials) conditions. To create an equal-opportunity territorial dispute [as done in Butler and Maruska, 2015], we divided the front compartment of the experimental tank into two parts using an opaque blue barrier placed perpendicular to the speaker barrier (Figure 2.2A). A quartered terracotta pot was placed on either side of the barrier so that a single territory was split by the barrier. Dominant males were identified from community tanks based on coloration (e.g. eyebars, bright yellow coloration) and display of stereotypical aggressive behaviors for > 1 week. One male was placed on each side of the experimental tank and allowed to acclimate for 2 days. Males were always size-
matched (within 10% of standard length) and fin clipped (middle or back of dorsal fin) for identification. On the morning of the trials, a video camera was set up in front of the tank. The sound file or control silence was started, and recorded for 5 min. The barrier was then removed and the pots repositioned to form a single territory that the two males fought over (Figure 2.2A). Each trial lasted 30 minutes from when the barrier was removed.

Figure 2.2. Experimental paradigm to induce aggressive and reproductive interactions. (A) During acclimation, the front experimental compartment was divided into two equal compartments, each housing a quartered terracotta pot and dominant male. After 2 days of acclimation, the barrier was removed and the pots repositioned to form a single territory over which the males fight. (B) The front experimental compartment housed a single halved terracotta pot to serve as a spawning territory for the dominant male. After 2 days of acclimation, a gravid female was added to the front compartment. In both set ups, the back compartment housed the underwater speaker (S) that was suspended from above the tank and hidden from view by a blue opaque barrier.

Videos were later scored by an observer blind to sound condition. We quantified stereotypical male aggressive behaviors, including lateral displays, frontal threats, bites, lunges, rams, and mouth fighting (Table 2.1). Behaviors were classified as either non-contact (e.g. lunge, frontal threat, lateral display) or contact (e.g. bite, ram) since use of non-contact behaviors is mediated by mechanosensory signaling (Butler and Maruska, 2015). Latency to begin fighting was defined as the time between when the pots were...
repositioned to when reciprocal aggressive behaviors were performed. Fight conclusion was determined based on criteria similar to that previously used (Butler and Maruska, 2015). The winner had to perform at least three dominance behaviors and either enter the pot at least three times in a 1-min period or stay in the pot for > 10 seconds. The loser had to fade his eyebar and other typical male coloration and perform submissive behaviors (e.g. flee, position of inferiority). Males will typically fight shortly after the barrier removal and have a single fight, after which one male emerges as the winner and spends the duration of the trials chasing and being aggressive towards the losing fish (Butler and Maruska, 2015). However, here, we observed that fights occurring during noise often occurred in bouts without the fight conclusion criteria being met. As such, we calculated fight duration based on the above criteria as well as the actual time spent fighting. A fight bout was considered over if neither fish performed a single aggressive behavior for > 30 seconds. Inter-bout-interval was calculated as the time from the last aggressive behavior to the next reciprocal exchange of behaviors. By subtracting the total inter-bout-interval time from total fight duration, we calculated the actual time spent fighting.

In addition to typical aggressive behaviors, we quantified freezing/stress behaviors. This was defined as the fish remaining stationary in the water and flaring all of their fins. Fish also had a dark eyebar and vertical banding on the trunk during this behavior. We also quantified the amount of time spent with the eyebar displayed. To measure mutual assessment, we quantified the time fish spent within one body length of each other without performing other behaviors (Butler and Maruska, 2015).
Table 2.1. Aggressive and reproductive behavior definitions. Some behaviors are typically only performed by males (M) while others are performed by both sexes (M/F). Behaviors are further classified as aggressive (A) or reproductive (R), but some behaviors are observed in both contexts.

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Definition</th>
<th>Sex</th>
<th>Context</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bite/Ram</td>
<td>With mouth open (bite) or closed (ram) one fish quickly hits flank of other fish</td>
<td>M</td>
<td>A</td>
</tr>
<tr>
<td>Lunge</td>
<td>Rapid forward movement towards other fish</td>
<td>M</td>
<td>A</td>
</tr>
<tr>
<td>Lateral display</td>
<td>Fish flares all fins, distends jaw, and gently vibrates body; often oriented perpendicular in front of other fish</td>
<td>M</td>
<td>A</td>
</tr>
<tr>
<td>Mouth fight</td>
<td>Two fish grasp jaws and gently push/pull</td>
<td>M</td>
<td>A</td>
</tr>
<tr>
<td>Frontal threat</td>
<td>While facing opponent, fish distends jaw and flares operculum</td>
<td>M</td>
<td>A</td>
</tr>
<tr>
<td>Chase/Flee</td>
<td>One fish rapidly swims behind the other</td>
<td>M/F</td>
<td>A/R</td>
</tr>
<tr>
<td>Pot entry</td>
<td>Fish enters into halved-terra cotta pot</td>
<td>M/F</td>
<td>A/R</td>
</tr>
<tr>
<td>Dig</td>
<td>Fish picks up gravel from inside pot and spits outside of the pot</td>
<td>M</td>
<td>A/R</td>
</tr>
<tr>
<td>Quiver</td>
<td>With anal fin displayed, fish rapidly vibrates body; dorsal fin often depressed against body</td>
<td>M</td>
<td>R</td>
</tr>
<tr>
<td>Tail waggle</td>
<td>Caudal fin exaggeratedly moved back and forth</td>
<td>M</td>
<td>R</td>
</tr>
<tr>
<td>Lead</td>
<td>Swimming in front of female and immediately swimming towards spawning territory. Often accompanied with tail waggle</td>
<td>M</td>
<td>R</td>
</tr>
<tr>
<td>Spawning</td>
<td>Male prods female urogenital opening to stimulate egg release, female picks eggs up into buccal cavity, then nips at male anal fin</td>
<td>M/F</td>
<td>R</td>
</tr>
<tr>
<td>Circling</td>
<td>Male prodding and female nipping behaviors, but no egg release</td>
<td>M/F</td>
<td>R</td>
</tr>
<tr>
<td>Time spent within 1 body length (BL)</td>
<td>Both fish within one body length of each other but not performing any behaviors</td>
<td>M/F</td>
<td>A/R</td>
</tr>
<tr>
<td>Aggressive-like behavior</td>
<td>Bite, ram, or lunge behavior directed at an object in the environment other than the fish</td>
<td>M/F</td>
<td>A/R</td>
</tr>
<tr>
<td>Stress flare</td>
<td>Fish stops swimming, flares all fins, and displays vertical black banding; jaw does not distend and no body vibrations present</td>
<td>M/F</td>
<td>A/R</td>
</tr>
</tbody>
</table>
2.2.4. Reproductive behavior protocol

To examine the impact of noise on reproduction, we induced reproductive interactions between a male and female during noise and control conditions (\(N = 6\) trials per condition). The abovementioned experimental tank and sound file was used to create a noisy environment. Dominant males were selected from community tanks, placed in the front compartment of the experimental tank (Figure 2.2B), and allowed to acclimate for 2 days. On the morning of the trials, an ovulated female was visually identified from community tanks based on a swollen abdomen, slightly distended jaw, protruding urogenital papilla, and actively courting males. Once the female was identified, the noise playback was started, and the female was quickly transferred to the front compartment of the experimental tank. A video camera was positioned in front of the tank and recorded for 30 min after the female was added to the tank.

We quantified stereotypical male courtship behaviors and female responses to each behavior (Butler et al., 2019). For males, we quantified the number of body quivers, tail waggles, and leads as overt courtship behaviors (see Table 2.1 for behavior details). We also quantified the number of digs (territory maintenance), and bites and chases directed at the females. For females, we qualified her behavior as ‘positive’, ‘negative’, or ‘no response’ to each male behavior or string of behaviors (see below for behavior string descriptions). If the female oriented towards or followed the male behavior within 1 sec, it was classified as a positive response. Negative responses were defined as orienting away from the male or swimming away from him within 1 sec of his behavior. No responses were classified by the lack of a positive or negative response. For both fish, we quantified the amount of time spent within the spawning
shelter (halved terra cotta pot) and against the front wall of the experimental tank, which was furthest from the speaker. The number of aggressive-like displays (e.g. bites, frontal threat) by the males to the back speaker wall or other tank component (e.g. air stone, filter) was also quantified. Finally, we quantified the number of circling and spawning bouts of the pair, as well as the time spent circling and spawning. During spawning, females release eggs on the substrate, pick them up into their mouth, and then nip at the male’s anal fin to induce sperm release. Then the male gently prods/nips at the female to release more eggs, creating a “circling” movement between the two fish where they alternate nipping at each other. Circling involves the same circular movements but does not involve egg release from the female.

Male *A. burtoni* are very behaviorally active during reproductive interactions and tend to perform multiple courtship behaviors within quick succession. For example, the most commonly seen courtship behavior sequence is a body quiver transitioned into a tail waggle that occurs as the male leads the female back to the pot. As such, in addition to quantifying individual behaviors, we also classified them as single behaviors, or 2-behavior, 3-behavior, or 4+behavior strings. To do this, we calculated the inter-behavior-interval and used a cutoff of 1 sec. Any behavior occurring within 1 sec of the previous behavior was classified as a string. Only overt courtship behaviors (i.e. quivers, waggles, leads, pot entries) were included in the string analysis.

**2.2.5. Auditory evoked potentials**

To determine how exposure to anthropogenic noise impacts hearing capabilities, hearing thresholds were measured using auditory evoked potentials (AEP) as done
previously (Maruska et al., 2012). Fish were anesthetized in 0.1% benzocaine in fish water, immobilized with an intramuscular injection of pancuronium bromide (~0.001 mg per gram body mass), and restrained in a mesh harness suspended from a PVC frame above the experimental tank on a vibration isolation platform (Figure 2.3). Fish were suspended in the center of the circular experimental tank (36 cm high, 30 cm diameter) and positioned just below the water surface and ~15 cm above the underwater speaker (UW-30) that was partially buried in gravel at the bottom of the tank. A gravity fed water system connected to a small tube in the mouth was used to ventilate fish during the experiments. Electrodes (stainless-steel sub-dermal electrodes, Rochester Electro-Medical, Inc., Tampa, FL) were sealed on the ends with nail polish so that ~1 mm of metal was exposed at the tip. A recording electrode was positioned in the dorsal musculature directly above the braincase, a reference electrode was placed beneath the skin between the eyes, and a ground wire was placed in the tank water.

Figure 2.3. Experimental set up used for auditory evoked potentials. (A) The aquarium was placed on an isolation platform, and a PVC frame was used to suspend the fish in water above an underwater speaker. (B-D) The fish was ventilated by a gravity fed water system. Recording (red) and reference electrodes (orange) were placed above the brain case and beneath the skin between the eyes, respectively (C), and a ground wire (green) was placed in the tank water. (D) Electrodes were coated with nail polish with ~1mm of metal exposed at the tip.
Sound stimuli were generated by a CED Micro3 analog to digital converter and attenuator, controlled with Spike2 software, amplified, and played through the underwater speaker. We tested 8 frequencies that encompass the hearing range of *A. burtoni* (Maruska et al., 2012): 100, 200, 300, 400, 500, 600, 800, and 1000 Hz. Each stimulus consisted of 2000 repetitions of 20 ms pulses (alternating phase) with an inter-pulse interval of 100 ms. For each frequency, stimuli were played at suprathreshold levels and decreased incrementally by 5 dB until an AEP response was no longer observed. Sound levels were calibrated by placing a hydrophone in the experimental tank at the position normally occupied by the fish head, presenting the sound stimuli (without phase alternation), and measuring the RMS voltage at each test frequency and intensity. AEPs were differentially recorded, amplified (10,000x), filtered (0.1-10 000 Hz) and then digitized by the CED A-D system. Threshold at each frequency was defined as the lowest sound level at which a repeatable AEP response was observed and power spectrum analyses (FFT, Hanning Window, 512 or 1024 points) showed peaks at twice the stimulus frequency (due to oppositely oriented hair cells).

Because gravid females have the best hearing sensitivity and are the primary intended receivers of acoustic communication, we only assessed the impact of noise on hearing capabilities in gravid females. Females were placed in the experimental tank and played the previously described noise file (as described in section 2.2.2; Figure 2.2) or control silence for 3 hours. Immediately after the 3-hour exposure, AEPs were performed. Only one control gravid female was run in the current experiment. Instead, auditory threshold values were compared to those already previously determined (Maruska et al., 2012). Four control fish (1 gravid female, 3 dominant males) were used
to verify that control threshold values were similar between the present study and those previously reported in *A. burtoni* (2-way RM ANOVA of dom. males: $F_{1,42} = 0.521, P = 0.789$), which validated their inclusion here for comparisons.

### 2.2.6. Statistics

All statistics were performed in SigmaPlot or IBM SPSS 25. Student’s t-tests (two-tailed) were used to compare data between the two sound conditions within behavioral condition. No outliers were detected via Grubbs outlier test. If data did not pass normality or equal variance testing, it was log, natural log, or square root transformed. If data still did not pass normality and/or equal variance, non-parametric testing was used. For comparison of aggressive and stress-related behaviors in aggression trials, we used a linear mixed model (LMM) because the two fish in a trial are not independent of each other. Winner or loser was a repeated within-subject factor, and sound condition (control vs noise) was a between-subject fixed factor. Individual subjects and trial ID were included as random effects and Tukey’s test was used to determine post-hoc differences. To compare the behavior strings used by the males during reproduction, we used a repeated measures ANOVA with the number of behaviors in the string as a repeated within-subject factor and sound condition as the between-subject factor. This was followed with Tukey’s post-hoc testing to isolate differences. To test for noise-induced hearing loss, a 2-way repeated measures ANOVA was used with frequency as the repeated within-subject factor and sound condition as the between-subject factor. Box plots are used throughout the text to represent data. The box extends to the furthest data points within the 25th/75th percentiles, and whiskers
extend to the furthest data points within 1.5x the interquartile range. Outliers (beyond 1.5x the interquartile range) are designated by open circles and are not reflective of statistical outliers. Data median is represented by a solid line and data mean by a filled circle.

2.3. Results

2.3.1. Noise exposure impacts fight timing but not overall aggressiveness

All seven control trials of the male-male interaction context resulted in a territorial fight that occurred shortly after the barrier removal. However, only seven of the nine noise trials resulted in a fight. Latency to initiate a territorial fight was longer in noise trials (12.472 ± 3.59 min) compared to control trials (2.679 ± 1.153 min) (Figure 2.4A; log transformed, $T_{14} = -3.298, P = 0.005$). However, during the longer latency time, fish did not perform increased mutual assessment behaviors (Figure 2.4B; $T_{14} = -0.742, P = 0.470$). Neither time spent fighting (Figure 2.4C; $T_{12} = 0.507, P = 0.621$) nor fight duration (Figure 2.4D; $T_{12} = -1.705, P = 0.114$) was different between sound conditions. During noise, fish fought in multiple bouts rather than in one succinct fight (Figure 2.4E; $T_{12} = -3.481, P = 0.005$). All control trials took place in one single fighting bout. In contrast, noise trials involved 4.286 ± 0.944 fighting bouts. The average time between fighting bouts was 43.950 ± 5.921 sec. The increased latency to fight and fight duration combines for a longer time to fight resolutions during noise (Figure 2.4F; log transformed, $T_{12} = -3.829, P = 0.002$).
Figure 2.4. Noise exposure alters male agonistic interactions. (A) Latency to begin fighting (30 min. max if no fight occurred) is longer when exposed to noise than control silence. (B) Prior to fighting, fish spend similar amounts of time performing mutual assessment (i.e. time within one body length of opponent). (C-D) Time spent fighting (i.e. cumulative bout duration) and fight duration (fight time and inter-bout-interval time) are similar in both sound conditions. (E) Fish fight in a single bout during control trials, but in multiple bouts during noise. (F) Due to longer latency to fight and fight duration, time to fight resolution is longer when noise is present. (G-H) Representative raster plots of individual aggressive behaviors (black vertical lines) demonstrates differences in fight structure between sound conditions. Different letters indicate statistical significance at $P < 0.05$. N = 7 control trials; 9 sounds trials total (A-B); 7 noise trials with fights (C-F). See methods for boxplot descriptions.

Although fight structure differed (Figure 2.4G-H) between the sound conditions, fish performed a similar number (Figure 2.5A; LMM; outcome: $F_{1,14} = 37.934$, $P < 0.001$; sound: $F_{1,14} = 1.886$, $P = 0.834$; outcome X sound: $F_{1,14} = 0.045$, $P = 0.834$) and type (Figure 2.5B; LMM; outcome: $F_{1,14} = 2.631$, $P = 0.149$; sound: $F_{1,14} = 5.105$, $P = 0.056$; outcome X sound: $F_{1,14} = 0.009$, $P = 0.925$) of aggressive behaviors between the sound conditions. Winners had a higher aggressive score than losers ($P = 0.001$) in both
sound conditions. In addition to aggressive behaviors, fish in the noise condition performed more stress behaviors, like freezing and flaring all fins, than fish in control trials independent of fight outcome (Figure 2.5C; LMM; outcome: $F_{1,14} = 0.523, P = 0.482$; sound: $F_{1,14} = 16.102, P = 0.001$; outcome X sound: $F_{1,14} = 0.624, P = 0.443$). Noise-exposed fish also spent more time with their eyebar displayed than control individuals (Figure 2.5D; LMM; outcome: $F_{1,14} = 28.691, P < 0.001$; sound: $F_{1,14} = 27.276, P < 0.001$; outcome X sound: $F_{1,14} = 29.912, P < 0.001$). In control animals, eyebar time was dependent on winning or losing the fight with winners displaying their eyebar more than losers, but outcome had no effect on eyebar time in noise trials.

Figure 2.5. Fish aggressiveness does not change, but noise-exposed animals perform more stress-related behaviors. (A-B) Fish have similar aggressive scores (number of aggressive behaviors per fight minute) and use contact (e.g. bites, rams)/non-contact (e.g. chases, lateral displays) behaviors similarly in control and noise conditions. Dotted line at 1 represents equal use of contact and non-contact behaviors. (C) Fish exposed to noise perform more stress behaviors (i.e. fin flaring, vertical banding, freezing) during
noise than control trials and (D) spend more time with their eyebar displayed. Different letters indicate statistical significance at $P < 0.05$. $N = 7$ control fish per group; 9 sound fish per group. See methods for boxplot description.

2.3.2. Noise affects female and male reproductive behaviors

Stereotypical male courtship behaviors were not impacted by noise. Males performed similar numbers of body quivers ($T_{10} = 1.439$, $P = 0.181$), tail waggles ($T_{10} = 0.607$, $P = 0.558$), leads ($U = 10$, $N = 12$ total, $P = 0.240$), and nips towards the female ($T_{10} = -1.500$, $P = 0.172$). Males also did not change their territory maintenance (digging out the territory; $U = 9.50$, $N = 12$ total, $P = 0.180$). While the total number of courtship behaviors did not change (Figure 2.6A; $T_{10} = 0.851$, $P = 0.415$), the location where the males performed these behaviors differed between the sound conditions. During noisy conditions, males performed more behaviors inside the pot (41.476 ± 10.449\%) compared to control conditions (12.300 ± 3.331\%; Figure 2.6B; $T_{10} = -3.708$, $P = 0.004$), but did not spend more overall time in the pot (Figure 2.6C; $T_{10} = 0.959$, $P = 0.360$).

Under silent conditions, males typically perform behavior strings in quick succession (e.g. body quiver, tail waggle, lead). To examine whether this was impacted by noise, we classified behaviors as occurring as a single event or in strings of 2, 3, or 4+ behaviors. Although the total number of courting events (after accounting for behavior strings) did not change (Figure 2.6D; $T_{10} = 0.192$, $P = 0.852$), males altered how they performed the behaviors in relation to other behaviors. During noisy conditions, males perform more single behaviors ($T_{10} = -5.647$, $P < 0.001$) than males in control trials (Figure 2.6E). However, males in control trials perform more strings of behaviors than males in noisy trials (2 behaviors: $T_{10} = 6.067$, $P < 0.001$; 3 behaviors: $T_{10} = 4.271$, $P = 0.002$; 4+ behaviors: $T_{10} = 2.304$, $P = 0.044$).
Figure 2.6. Males perform a similar number of courtship behaviors between noise and silent conditions, but how and where they use them changes during noise. (A) Males exposed to control silence and noise produce similar numbers of overt courtship behaviors. (B) During noise, males perform more behaviors inside the spawning shelter but (C) do not spend more time in the pot compared to silence. (D-E) Although the total number of courting events does not change, males exposed to noise perform more single behaviors and less 2, 3, and 4+ behavior strings than males in control conditions. (F) Males exposed to noise perform aggressive-like behaviors (i.e. biting, frontal threats) towards tank objects, such as the air stone and speaker wall. However, males in control trials did not perform any of these aggressive-like behaviors except towards the female. Different letters indicate statistical significance at $P < 0.05$. $N = 6$ for all. See methods for boxplot descriptions.

In addition to changes in courtship behaviors, males exposed to noise performed aggressive-like behaviors towards the back wall (behind which the speaker was housed) or other tank object (i.e. air stone, filter). No control males performed these aggressive-like behaviors to the back wall, but all noise-exposed males did (Figure 2.6F; $U = 3.00$, $N = 12$ total, $P = 0.015$). However, stereotypical male aggressive behaviors directed at the female (bites, chases) did not differ between the two groups.
\( U = 14, N = 12, P = 0.589 \), and these behaviors could be considered as part of the early courtship behavioral repertoire rather than aggressive.

Females were less responsive to male courtship behaviors when noise was present. Female positive responses to male behaviors (i.e. following them or orienting towards them) was lower during noisy conditions (Figure 2.7A; \( T_{10} = 5.018, P < 0.001 \)). Females positively responded to \( \sim 50\% \) (48.719 \( \pm \) 7.901\%) of male courtship events in control trials, but this was reduced to less than 10\% (6.833 \( \pm \) 2.676\%) during noise. Females also entered the pot less often during noise (Figure 2.7B; \( T_{10} = 2.292, P = 0.045 \)), but spent a similar amount of time in the shelter (Figure 2.7C; \( T_{10} = -0.584, P = 0.572 \)). This was because females often entered the pot near the beginning of the noise trials and stayed there, instead of revisiting multiple times throughout the trial. Noise-exposed females spent more time at the front wall of the tank (as far from the speaker as possible) than control females (Figure 2.7D; \( U = 5.5, N = 12, P = 0.041 \)).

Figure 2.7. Females are less responsive to male courtship behaviors during noise. (A) Females perform fewer positive responses to male courtship behaviors. (B-C) Females enter the spawning shelter less during noise but spend a similar amount of time there compared to control trials. (D) During noise, females spend more time at the front wall of the experimental tank, far away from the speaker. Different letters indicate statistical significance at \( P < 0.05 \). \( N = 6 \) for all. See methods for boxplot descriptions.
Circling behaviors (no egg laying) of the male-female pair occurred in all control trials (Figure 2.8A). However, only 67% (4 of 6) of noise trials contained circling. In addition, spawning occurred in 67% (4 of 6) of control trials but only in 1 (17%) of the noise trials. Circling behaviors always preceded spawning behaviors. The latency to initiate circling was longer during noise trials compared to control trials (Figure 2.8B; $T_{10} = 2.593, P = 0.029$). In trials where circling and/or spawning occurred, the number of circling/spawning events (Figure 2.8C; $T_{10} = 1.739, P = 0.113$) and the time spent circling/spawning did not differ between the sound conditions (Figure 2.8D; $T_{10} = 0.232, P = 0.823$).

Figure 2.8. Fish perform less late-stage consummatory reproductive behaviors during noise. (A) Four of 6 control trials resulted in spawning while the other two involved circling behaviors. In contrast, only 1 of 6 noise trials involved spawning, 2 included circling behaviors, and 3 had neither. (B) The latency to first circling bout (circling always preceded spawning) was longer in noise trials. (C-D) Despite fewer trials involving circling/spawning, when it did occur, circling/spawning bouts and time engaged in circling/spawning was not different between the sound conditions. Different letters indicate statistical significance at $P < 0.05$. $N = 6$ for all. See methods for boxplot descriptions.

2.3.3. Noise exposure impairs gravid female hearing capabilities

Under normal conditions, gravid females typically have best hearing sensitivity between 200-300 Hz, which corresponds to peak frequencies of male courtship sounds
(Maruska et al., 2012). There was an overall effect of frequency ($F_{7,67} = 28.474; P < 0.001$), but not sound condition ($F_{1,67} = 0.480; P = 0.504$) on hearing threshold recorded by AEPs in gravid females. However, the effect of sound condition was dependent on frequency ($F_{7,67} = 2.555; P = 0.022$). Noise-exposed gravid females have significantly higher thresholds (i.e. lower sensitivity) at 200 and 300 Hz compared to control females ($P = 0.016$, $P = 0.034$). There is no noise-induced threshold shift at 100, 400, 500, 600, or 800 Hz ($P > 0.1$ for all). Noise-exposed females were tested at 1000 Hz, but control females were tested at 1100 Hz, so this high frequency was not compared between sound conditions.

Figure 2.9. Noise-exposed gravid females have lower hearing sensitivity at 200-300 Hz. (A) Both control and noise-exposed gravid females have peak hearing thresholds at
200-300 Hz, but noise-exposed females have significantly higher thresholds (i.e. worse hearing) at 200 and 300 Hz compared to control females. Noise exposure did not impact thresholds at any other frequency. (B-C) Example of auditory evoked potential traces recorded from control (B) and noise-exposed gravid females (C) to 200 Hz stimuli (purple bottom trace). Threshold was determined as the lowest intensity that produced a repeatable waveform and the presence of an FFT peak at twice the stimulus frequency. For 200 Hz, threshold (dashed boxes) in these examples was set as 105 dB and 115 dB for control and noise-exposed gravid females, respectively. * indicates significant difference within frequency between control and noise-exposed gravid females.

2.4. Discussion

Anthropogenic noise is pervasive in almost all aquatic and terrestrial environments and can have severe detrimental impacts on site-attached animals that are unlikely to leave their territory even in unfavorable conditions. Despite its crucial role in species persistence, there exists a paucity of information on how noise impacts social behaviors and communication. We found that while noise did not fully deter social interactions from occurring, territorial fights and circling/spawning were less likely to occur during noise. Noise also changed how and where fish performed social behaviors. For example, instead of territorial fights occurring in a singular fight, they occurred in multiple bouts. During reproductive interactions, males performed more of their behaviors inside of the spawning shelter, and as such, females were less responsive to male courtship. Male behaviors also occurred as more singular events, instead of stringing together multiple behaviors. Behavior strings may encode information differently than single behaviors, such that a male performing 4+ behaviors in close succession may appear stronger or more fit than a male performing only single behaviors. In both territorial and reproductive interactions, fish performed more stress-like behaviors, such as freezing and flaring fins, hiding along the front wall of the tank, and biting inanimate objects. Together, these data suggest that underwater noise has
negative impacts on social communication and behaviors in both territorial and reproductive contexts within a single fish species.

The ability to defend one’s territory from rival males is vital to reproductive success. Like many territorial animals, male *A. burtoni* use their territory for reproduction, feeding, and protection. Non-territorial males are reproductively repressed and have little to no opportunity to spawn with females (Maruska, 2014). Importantly, males still defended their territory from rival males, even during noise; however, they took longer to initiate a fight. This increased latency could relate to changes in cost-benefit analysis. For example, it is possible that the high background noise diminishes the quality of the territory (Brumm, 2004), making it less important to defend. The risks associated with a costly and dangerous territorial fight could outweigh the resource benefits of the territory. During aggressive interactions, *A. burtoni* males did not change the number or type of aggressive behaviors. In contrast, in the cooperatively breeding cichlid *Neolamprologus pulcher*, anthropogenic noise resulted in fewer digging (territory maintenance) behaviors (Bruintjes and Radford, 2013). Subordinate individuals also received more aggression from dominant fish, but the effects on aggression were both sex and context specific. Instead of changes to individual behaviors, we found that male-male fight structure was significantly altered during noise. While we cannot tease apart the specific reason fish switched from fighting in a single fight to multiple bouts during noise, one possibility is that the noise serves as a stressor and/or distraction. This is reflected in the higher number of stress behaviors, which were most commonly observed during the inter-bout-interval time. The changes in fight behaviors observed, especially the increased time to fight resolution, can have negative impacts on anti-
predator behaviors. First, engaging in a territorial fight makes an individual less aware of their surroundings, as does the types of behaviors being performed. South American cichlids, *Nannacara anomala*, were slower to detect approaching predators when engaged in contact behaviors compared with non-contact behaviors (Jakobsson et al., 1995). In addition, anthropogenic noise can act as a further distraction and increase mortality due to predation (Simpson et al., 2016b). Fathead minnows (*Pimephales promelas*) were less likely to respond to conspecific alarm (chemosensory) cues during noise (Hasan et al., 2018), and the Caribbean hermit crab (*Coenobita clypeatus*) allowed a simulated predator to get closer before noticing it (Chan et al., 2010). These noise-induced changes in anti-predator behaviors can have major fitness consequences.

Anthropogenic noise is particularly pervasive in shallow shore areas, which unfortunately corresponds to where many territorial fish live. Of the over 800 species of fish that are known to produce sounds, most produce sounds during reproduction. These sounds can encode vital information about the sender’s sex, reproductive state, social status, size, and motivation, but are typically only intended for close-range (< 1 m) communication. Although male *A. burtoni* did not change the number of courtship behaviors performed, they did change how and where they performed these behaviors. A sender must survey their environment and determine whether any factors may interfere with signal transmission and modify it as needed (Cole, 2013). To do this, senders may change the location, timing, type, or sensory channel of the signal to maximize probability of detection. However, senders must also account for the energetic requirements of producing the signal, and if the costs outweigh the potential benefits,
may choose not to engage in social communication at all. For example, it is the sender’s responsibility to position their visual displays in a way that will maximize visibility to the receiver. In our reproductive context, this means dominant males are responsible for positioning their courtship in a way that increases the probability of female detection and response. In the natural environment and in our reproductive control trials, males often swim directly up to or in front of a female to produce a body quiver (with associated courtship sound) and tail waggle. This close-range communication helps to ensure females will detect and appropriately respond. However, when noise was present during reproductive trials, males performed more behaviors inside of the spawning shelter instead of adjacent to females. Without displaying in front of the females, they are unlikely to see and respond to these visual signals. The water movements associated with body quivers and tail waggles likely stimulate the female lateral line system (Butler and Maruska, 2016b), but mechanosensory information only transmits ~1-2 body lengths from the fish. While the acoustic courtship sound produced during body quivers is likely audible at this short distance, the increased noise could mask the sound all together or alter its characteristics. Thus, by males simply changing the location of the courtship displays, they are likely removing or altering visual, mechanosensory, and auditory signals intended to impress females.

We found that noise-exposed gravid females had higher auditory thresholds, indicating worse hearing, at 200 and 300 Hz compared to control gravid females. This threshold shift corresponds to the frequency range gravid females are most sensitive and a dominant frequency component of male courtship sounds (Maruska et al., 2012). After accounting for this threshold shift, noise-exposed gravid female hearing
capabilities more closely resembles that of brooding females. Noise-exposed females were also much less responsive to male courtship attempts. This breakdown in communication is likely a compounded effect of background noise masking acoustic signals and males altering the location of multisensory signal production. Based on this, it is not surprising that spawning rates are lower in noisy conditions. It is possible that A. burtoni males could increase the number of courtship sounds (higher incidence with body quivers) in order to maximize acoustic signaling, although this was not tested because recordings were too noisy to distinguish the low intensity courtship sounds from noise playback, indicating that noise exposure likely masked or altered male courtship sounds. Taken together, these data indicate that background noise affects all three components of social communication: signal production by the male, signal detection capabilities by the female, and potential masking of the signal itself.

When one sensory modality is disrupted, aside from ceasing communication altogether, two possible adjustments exist. First, animals can change how, when, and where they produce their signals to maximize receiver detection and response. For acoustic communication in fishes, this is not always possible. Fish can change temporal aspects of their calls (i.e. produce sound during low-noise times) or increase the number and duration of calls, but physiological constraints inherent in the mechanisms of sound production typically prevent fish from being able to adjust the frequency or amplitude of their calls [(Radford et al., 2014), but see (Holt and Johnston, 2014; Luczkovich et al., 2016)]. In contrast, birds, frogs, and mammals are known to adjust the amplitude, pitch, repetition rate, and duration of notes during abiotic noise [e.g. (Grafe et al., 2012; Ríos-Chelén et al., 2015)]. An alternative strategy to modulating the disrupted
channel is to instead switch channels to a less disturbed one. These cross-modal changes due to noise are observed in several species of fishes. Noise had no effect on nest building in either the two-spotted goby (*Gobiusculus flavescens*) or painted goby (*Pomatoschistus pictus*) (de Jong et al., 2018a). However, both species decreased the number of drumming behaviors but not the number of thumps. Interestingly, in two-spotted gobies, there was no change in visual displays, but painted goby males decreased their visual displays during noise. This demonstrates that even in closely related species, noise can have different effects. While noise decreased the number visual and acoustic displays by male painted gobies, it also changed the female’s preference for visual and acoustic signals (de Jong et al., 2018b). Under control conditions, a female’s preference was predicted by the number of male acoustic displays. However, when noise was added, females instead relied on visual displays for mate choice. Similar to our results in the cichlid, painted gobies had decreased spawning rates during noise (de Jong et al., 2018a). Aquatic invertebrates such as cuttlefish (*Sepia officinalis*) also suffer from noise induced effects across multiple sensory modalities (Kunc et al., 2014) by increasing their visual displays. Importantly, the authors of that study note that these cross-modal changes in visual behaviors can help mitigate the negative impacts of noise but do not completely compensate. This is especially true in species that use non-redundant signaling in which signals in different sensory channels provide receivers with different types of information (Johnstone, 1996; Partan and Marler, 1999). Both male and female *A. burtoni* are known to contextually release their urine (containing putative pheromones) in the presence of threats or reproductive opportunities (Field and Maruska, 2017; Maruska and Fernald, 2012).
Because this species can control when and where they release their urine, future studies should test for cross-modal impacts of noise on chemosensory signaling. Combined with our data, this highlights the importance of considering the natural multimodal nature of social interactions and possibility of cross-modal changes due to noise.

Cross modal impacts of noise were not restricted to reproductive contexts. Male *A. burtoni* spent more time displaying their eyebar during noisy trials. Males displaying an eyebar are behaviorally more likely to attack another male, and conversely, males with an eyebar are more likely to be attacked (Leong, 1969). As such, visual display of the eyebar is an essential component of male-male aggressive interactions (Heiligenberg et al., 1972). Under control conditions, both males displayed their eyebar at the beginning of the trial. As the fight progressed, the losing fish lost his eyebar while the winner maintained it for the duration of the trial. However, both the fight winner and loser spent equal time with the eyebar displayed during noise, even after the conclusion of the fight. Eyebar “on” is the default state (Muske and Fernald, 1987), so this increased display of the eyebar could relate to not turning the eyebar “off” due to stress or other energetic demands. During periods of stress, males typically get vertical banding along their trunk, and often times have their eyebar displayed, but the eyebar was displayed in noise-exposed males even when vertical banding was absent. Although we are unable to determine if the increase in eyebar displays is a by-product of stress or an intentional signal, it ultimately results in a similar outcome: an increased visual display of dominance. Perhaps this increased visual cue, even during non-fight times, could explain why fight structure was changed. Instead of turning their eyebar off
at the conclusion of a fight, the eyebars remain on, leading to continued fighting and aggression between the two males.

Anthropogenic noise is a global pollutant and affects most aquatic and terrestrial ecosystems. Changes to natural soundscapes are limiting communication space and affect many life-history stages. While traditional studies on noise exposure focused on major organ damage, mortality, and other dramatic impacts, recent research has focused on sub-lethal impacts of noise. By examining these subtle changes in behavior, physiology, and communication due to noise exposure, we can identify earlier indicators of noise susceptibility. These subtle changes could be more important for management and conservation efforts across a wide range of species moving forward.

2.5. Summary

Changes in social communication can have dramatic impacts on sexual selection and mate choice. As evidenced in gobies, noise in one sensory modality can shift the relative importance of signals in other channels and even drive loss of certain signals (de Jong et al., 2018b). When testing for anthropogenic effects on social behaviors and communication, we should focus on all three components of communication: the production of the signal (behavior), transmission of the signal (environment), and the receiver’s physiology (ability to detect the signal) and their behavioral response. More studies are needed to examine noise-induced impacts on signal production (timing, location) before we can fully understand the determinantal impacts anthropogenic noise can have on animals. All together, these studies highlight the species, sex, and context-
specific effects of anthropogenic noise on a social, territorial, and relatively site-attached fish.

2.6. References


3.1. Introduction

The ability to detect and accurately perceive signals that convey information about the sender’s sex, reproductive state, motivation, and identity are important for many vital life functions, including predator defense, territoriality, and reproduction. Unfortunately, human activities generate increasing amounts of sensory pollution in ecosystems across the world with detrimental effects on both terrestrial and aquatic animals (Halfwerk and Slabbekoorn, 2015). Underwater anthropogenic noise has risen rapidly in the past century due to increases in pile driving, sonar use, and shipping travel, which has intensified ambient underwater noise levels by over 30 dB in the frequency range that most fish produce and detect acoustic stimuli (Board, 2005; Crovo et al., 2015; Purser and Radford, 2011; Radford et al., 2014; Scholik and Yan, 2001; Scholik and Yan, 2002a; Scholik and Yan, 2002b; Vasconcelos et al., 2007). Fishes depend on their auditory system for anti-predator behaviors, prey detection, orientation, and social communication (Cole, 2013). Aquatic anthropogenic noise is linked to changes in feeding and foraging behaviors (Bruintjes and Radford, 2014; Picciulin et al., 2010; Purser and Radford, 2011), decreased growth rates (Anderson et al., 2011; Lagardère, 1982), and damage to the sensory hair cells in the inner ear (Smith et al., 2006). Elevated and persistent noise can also induce stress (Smith et al., 2004; Wysocki et al., 2006), further interfering with an animal’s ability to feed, reproduce, care for young, evade predators, and navigate their environment.
Parental care life history stages are particularly sensitive to perturbations (Wong and Candolin, 2015), and fish engaging in parental care behaviors are often at greater risk than fish species with other reproductive strategies (Parent et al., 1995). Parental care (post-fertilization behaviors intended to promote offspring survival) occurs in approximately 22% of teleost fishes (Blumer, 1982; Gross and Sargent, 1985), and can vary from nest defense, to egg fanning, to feeding and cleaning, to mouthbrooding. Most fish species that provide parental care live in shallow, nearshore areas (Blumer, 1982) that are subjected to high amounts of anthropogenic disturbances from recreational and commercial boating and other activities. As such, anthropogenic noise may be particularly detrimental to fishes engaged in parental care behaviors and to early-life developing individuals, particularly to species that are site-attached and unlikely to leave a noisy environment. The impairment of parental care behaviors may have direct negative impacts on the developing offspring, and ultimately result in decreased reproductive fitness.

Mouthbrooding is an extreme form of parental care in which one fish carries the developing embryos/larvae for the full or partial duration of development inside their buccal cavity (Oppenheimer, 1970). Mouthbrooding often results in the brooding fish undergoing forced starvation for an extended amount of time and has evolved independently in several groups of fishes (Oppenheimer, 1970). While brooding increases the likelihood of larvae hatching and success, it is costly and stressful for the brooding parent fish due to the physiological demands. To date, no study has examined the impact of noise on mouthbrooding fishes. Mouthbrooding fishes exposed to noise are not only themselves susceptible to noise-induced changes in behavior and
physiology, but their brood can also be directly affected by the noise. In addition, the developing fish may suffer from indirect consequences due to effects on the brooding parent. For example, developing larvae can feed on the mucus inside the buccal cavity, which indicates some form of maternal-embryo nutrient transfer (Kishida et al., 2000) and potential transfer of immunity (Sin et al., 1994). Investigating the impact of noise on mouthbrooding fishes is of extreme importance. Any disruption to mouthbrooding, from the parent or offspring level, can have devastating effects on species persistence.

Although the impact of anthropogenic noise on fish behavior and physiology has become a prevalent research topic in recent years, only a handful of studies have examined how larval fishes may cope with anthropogenic sounds, and no study has examined how noise exposure during the mouthbrooding period influences the young after they are released. Past studies found mixed results on the effects of noise on growth and development in fishes. For example, hatching success and growth was not affected by noise-playback in the substrate spawning African cichlid Neolamprologus pulcher (Bruintjes and Radford, 2014). Similarly, rainbow trout (Oncorhynchus mykiss) raised in noisy and silent conditions had similar growth and survival rates after two months, but fish raised in noisy conditions had slower growth rates for the first month (Davidson et al., 2009; Wysocki et al., 2007). However, seismic air guns caused up to 100% mortality in lake trout (Salvelinus namaycush) larvae (Cox et al., 2012), and noise exposure to Atlantic cod (Gadus morhua) larvae impacted growth, use of yolk sac, condition factor, and ability to avoid predators (Nedelec et al., 2015). The reason for these varied results could be due to differences in exposure protocol, species variability due to hearing or other physiological differences, or timing of the exposure. However,
the majority of these studies found some detrimental impacts of noise during early-life stages. In addition to affecting growth and health, noise exposure impacts crucial behaviors aiding survival. Boat noise impairs orientation and settlement behaviors in coral reef fish larvae (Holles et al., 2013; Simpson et al., 2016a), and several studies demonstrated that noise exposure can impair shoaling and schooling behaviors (Herbert-Read et al., 2017; Sarà et al., 2007). Shoaling is a natural behavior observed in approximately half of all fishes when individuals stay in close proximity to each other but do not need to be polarized in a common direction as in a school (Radakov, 1973). In addition, shoaling is thought to enhance protection from predators and increase foraging efficiency (Pitcher et al., 1982), and a fish’s motivation to shoal is affected by the quality (e.g. presence of parasites, nutritional condition) of their potential shoalmates (Barber et al., 1998; Krause and Godin, 1996; Krause et al., 1999). Further, noise exposure affects learning/cognition and attention, with animals often distracted from their typical behaviors (Ferrari et al., 2018). While anthropogenic noise can directly cause physical injury and mortality, the effects on behavior can be equally devastating but are understudied.

In the mouthbrooding cichlid fish *Astatotilapia burtoni*, females incubate developing embryos/larvae in their buccal cavity for ~14 days. In larval *A. burtoni*, the otoliths develop ~5 days post fertilization (dpf) suggesting that larvae in the buccal cavity may be able to detect acoustic stimuli after this time. Although we do not know for sure that *A. burtoni* fry can detect sounds, 12 dpf fry have neural activation (measured via a neural activation marker, pS6) in auditory processing regions of the brain
(personal observations). This suggests developing juveniles are able to detect acoustic stimuli and may be susceptible to impacts of acoustic overstimulation. Here, we tested the hypothesis that noise exposure impacts the mother and developing young in a maternal mouthbrooding cichlid fish. Specifically, we tested the following predictions: (1) exposure to noise during brooding will impair mouthbrooding behaviors, and (2) exposure to noise during development will negatively impact growth, mortality, and behaviors of juveniles. We found that noise-exposed mouthbrooding females had decreased brooding success due to increased cannibalism and premature brood release. In addition, post-release maternal care behaviors were also decreased. Juveniles exposed to noise during development had lower condition factors and higher mortality. They also had altered shoaling behaviors, increased freezing behaviors, and a delayed onset of adult-typical territorial behaviors/colorations. Together, these data indicate that noise exposure during crucial parental care stages can have dramatic and devastating impacts on reproductive fitness.

3.2. Materials and methods

3.2.1. Experimental animals

*Astatotilapia burtoni* were bred under laboratory conditions from a wild-caught stock. Community aquaria contained 10-20 adults and were maintained at conditions mimicking natural environments (pH=7.6-8.0; 28-30°C; 12 L:12 D diurnal cycle). Adults were fed cichlid flakes daily and supplemented with brine shrimp twice weekly. Each community contained several halved terracotta pots to serve as spawning territories. Community fish were monitored daily for the presence of mouthbrooding females, which
were identified by the presence of a distended jaw (due to fertilized eggs in the mouth).

All experiments were performed in accordance with the recommendations and guidelines stated in the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, 2011. All animal care and collection were approved by the Institutional Animal Care and Use Committee (IACUC) at Louisiana State University, Baton Rouge, LA.

3.2.2. Noise exposure protocol

To examine the impact of anthropogenic noise on maternal care behaviors and juvenile development, mouthbrooding females (with developing young in buccal cavity) were randomly assigned to either the control or noise sound treatments. Experimental tanks (glass; 38 L; 49.5 cm L x 25.4 cm W x 29.2 cm H; Fig. 3.1A) were placed on several layers of foam insulation to isolate them from any potential vibrations from the environment. Each tank was divided into two compartments by an acrylic divider (length = front: 35 cm; back: 14.5 cm). The back compartment contained a submerged underwater speaker (UW-30) behind a blue opaque barrier. The speaker was placed in a separate compartment and hidden from view to prevent fish from interacting with it. The speaker was suspended from above so that no part of the speaker (including the wire) touched any part of the tank. The front compartment contained a single halved terracotta pot ~20 cm in front of the speaker. The same experimental setup was always present for both control and noise conditions.

On the morning of the seventh day of brooding, mouthbrooding females were quickly netted from community tanks and placed in the front compartment of the
experimental tank described above. After a short acclimation (15 min), fish were exposed to their assigned sound condition for 3 h and allowed to recover in silence for 30 min before being placed in isolation in a 38 L recovery tank. The noise group was exposed to increased background noise via a sound file played through the underwater speaker. The sound file created in Audacity (Version 2.1.1 https://audacityteam.org/)

Figure 3.1. Experimental set up for noise exposure. (A) Summary of experimental timeline illustrating periods of mouthbrooding females monitored in community tanks (1), sound treatment (2), and post-treatment isolation until fry release (3). Mouthbrooding females were monitored in community tanks (1). In the morning of the seventh day of mouthbrooding, females were placed in the experimental tank and exposed to either noise or silence for 3 hours (2). They were then transferred to an isolation tank (3) and monitored until fry release. (B) Example spectrogram of noise playback file comprised of random tones ranging from 100-2000 Hz for varying intervals of time. Warm colors (orange, pink, red) indicate higher power while cool colors (blue and purple) represent weaker power. Light grey indicates the predominate frequency. S: underwater speaker. Scale bar in B = 5 sec.
was comprised of pure tones ranging from 100-1500 Hz and lasted ~5 min in length but was played on a loop for a total of 3 h. Tone order and duration (0.5-4.0 s) were randomized. The computer-generated sound file was amplified (TOA, CA-160) before being played through the speaker. A hydrophone was placed at various locations in the front compartment to record sound pressure levels of the sound playback. The amplifier was adjusted until the average sound pressure level (SPL) was ~140 dB re: 1 μPa just above the territory. The second group of fish was exposed to silence. While this does not represent complete silence (faint hums from water filters and air pumps on nearby tanks), the average SPL recorded in the tank was much lower (~110 dB re: 1 μPa). In this condition, the computer, amplifier, and speaker were all still present and turned on, but no sound file was selected for playback.

3.2.3. Maternal care behaviors and juvenile health and development

After being transferred to isolation tanks, mouthbrooding females were observed daily for the presence of prematurely-released fry or potential cannibalism. Once fry were released, the female was “threatened” by an observer quickly approaching the tank to examine if she would provide parental care by taking them back into her buccal cavity, a normal maternal response that typically lasts for ~1-3 days following fry release. Following this test, the brooding female was quickly removed from the tank and measured for standard length (SL) and body mass (BM). The day and time of release, presence or absence of parental care behaviors, and number of juveniles were recorded. To examine juvenile size upon release, 3-4 fry were randomly selected and collected on the release day (day 0) and measured for SL and BM. Remaining juveniles
were allowed to develop and grow, and then 3-4 individuals per brood were randomly selected and collected on days 7, 14, 21, and 28 days post-release (dpr) prior to feeding and measured for SL and BM. Fulton’s condition factor was calculated as $BM/(SL^3)$ for each fish. All collected fish within a brood were averaged together to get a single value for each brood at each time point. Fish were fed 5% of their BM in crushed cichlid flakes daily for the first two weeks followed by a reduction to 3% of their BM in crushed cichlid flakes daily. Juveniles were monitored daily for mortality and onset of adult-typical behaviors and coloration. Each morning (8-10 am), an observer watched the fish for 10 min and recorded if any fish displayed coloration (e.g. eyebars, yellow coloration, etc) or territorial behaviors, such as chasing or biting other juveniles.

Because all animals were allowed to live for months after the exposure, we did not collect serum for cortisol measurements, and we opted against waterborne assays because the treatment protocol and handling was already stressful for the fish and we did not want to exacerbate this effect. In addition, cortisol measurements in this species are quite variable, and mounting evidence across taxa suggests that it is not the most reliable measure of a stress response (Breuner et al., 2013; Maruska and Fernald, 2014b).

3.2.4. Startle/stress response protocol

To examine how noise exposure during development might impact startle responses in A. burtoni juveniles, we examined startle behaviors at 14 and 28 days post-release (dpr). Ten fry per brood were placed in a 38 L aquarium with no shelter. After a 10min acclimation period, a padded hammer was gently tapped against the
outside of the tank producing a ~130 dB re: 1 μPa acoustic stimulus. The hammer was suspended from a pendulum and raised to the same level so that it consistently produced the same stimulus. Without high speed cameras, we were unable to examine the true startle response documented in many fishes, including cichlids, which occurs in milliseconds and is mediated by hindbrain Mauthner neurons (Canfield and Rose, 1996; Canfield, 2003). Instead, we were interested in the amount of time juveniles spent stationary, or “freezing time” after an acoustic startle. Across taxa, freezing behaviors, or time spent motionless within an arena or aquarium, is used as a measure of stress, with increased time motionless/stationary correlated with higher stress (Koolhaas et al., 1999). Here, we used the latency to return to normal swimming after an acoustic startle as a proxy for this behavior. A video camera (Canon HFR400) recording at 60 frames per second was placed immediately above the aquarium and recorded for 5 min after the acoustic startle. Videos were later analyzed by an observer blind to brood treatment identity. The first frame of the acoustic stimulus marked the “start time”. The video was then slowly scanned to determine when at least 5 fish (50%) resumed swimming.

3.2.5. Shoaling behavior analysis

Juvenile *A. burtoni* shoal when placed in a large, open space, and we measured shoaling behavior in control-silent and noise-exposed broods at 14 and 28 dpr. A grid (2 cm X 2 cm, black lines on white background) was placed under a 38 L aquarium for distance measurements. During both acclimation and trial, the aquarium was filled to a depth of 10 cm to ensure fish spread out horizontally and not vertically in the water column. Due to constraints of brood size and high mortality in the noise exposure group,
we used only ten juveniles per brood to examine shoaling behavior. In one noise exposure group, less than ten juveniles were present by the 28 dpr trial, so this brood was excluded from this time point. After a 15 min acclimation time, video was recorded from immediately above the tank for 10 min. The video was later analyzed by an observer blind to brood treatment identity. All measurements were performed in Image J (imagej.nih.gov/ij/). We calculated (1) average distance to nearest neighbor, (2) average distance between fish, and (3) shoaling density. For distance measurements, a point was placed on the junction between the head and trunk of the fish, at the origin of the dorsal fin. A line was then drawn between these points and distance recorded (Fig. 3.2). For shoaling density, we calculated the number of fish within a randomly selected 150 cm² region of interest. Each of the above measurements were taken for five randomly selected frames within the 10 min video and averaged together for each individual.

Figure 3.2. Example of distance measurements for shoaling analysis. A point was placed on the junction between the head and trunk of the fish, at the origin of the dorsal fin. For distance measurements, a line was drawn between these points on neighboring fish and distance (D) recorded. For example, DF1-F3 is the distance (in mm) between F1 and its nearest neighbor F3. For F1, the distance between fish is the distance between itself and F2 (DF1-F2) and F3 (DF1-F3). F1, F2, F3: fish 1, 2, and 3 respectively.
3.2.6. Statistics

All statistics were performed in SigmaPlot 12.3. Student’s t-tests were used to compare data between the two sound conditions when not measured across time. Since data points were collected from the same brood of fish weekly (growth and mortality) and biweekly (shoaling and freezing time), a 2-way repeated measures ANOVA was used. Treatment (control vs noise) and week (0, 7, 14, 21, 28 dpr) were fixed factors with brood identity as the repeated variable. ANOVAs were followed by Tukey’s post-hoc testing. All data were checked for outliers using Grubbs outlier test, but none were detected, and normality and equal variance were met with all data sets. Mean ± standard deviation is represented by closed circles and error bars in each figure.

3.3. Results

3.3.1. Noise impacts brooding success and maternal care

Mouthbrooding females typically carry developing broods for 10-14 days. Of the ten control-silence fish, nine released within this window and the tenth fish died (Figure 3.3A). In contrast, only one of the 12 noise-exposed brooding females released her brood within the normal time frame (Figure 3.3B). Of the remaining noise-exposed females, four released between 14-18 days post fertilization, three pre-maturely released underdeveloped fry (with un-resorbed yolk sacs) shortly after noise-exposure, and four cannibalized their brood (verified by dissection). As such, control females had 90% successful broods, but noise-exposed females had only 41.67% success (Figure 3.3C). Of the successful broods, control females held their broods for significantly less time than noise-exposed brooding females (Figure 3.3D; \( t = -5.557, df = 12, P < \))
0.001). Of the nine successful control brooding females, 100% performed maternal care behaviors for the first 1-2 days after fry release by taking the juveniles back into the buccal cavity when threatened. However, only 60% (3 of 5) of noise-exposed brooding females performed this same maternal care behavior. There was no difference in the size of the broods at release (Figure 3.3E; $t = 0.009$, $df = 12$, $P = 0.993$) with an average of 22 fish in each brood.

Figure 3.3. Exposure to noise during mouthbrooding impairs maternal care. (A) Most control females released their broods within the normal timeframe (10-14 dpf). (B) However, noise-exposed females had a greater incidence of brood cannibalization (green; 33%) and premature release (pink; 25%). Release early: <10 dpf; Normal: 10-14 dpf; Release late: >14 dpf. (C) Mouthbrooding females exposed to noise had a 42% brooding success rate while control females had a 90% brooding success rate. (D) Of successful brooding females, noise-exposed females held onto their brood for significantly longer than control females. (E) Brood size did not differ between control and noise-exposed broods. $N = 10$ control and 12 noise brooding females, but only 9 control and 5 noise females released broods for measurements in D and E. In D and E individual data points are plotted as unfilled circles with mean ± s.d. plotted to the side of each group. Different letters indicate statistical significance at $P < 0.05$.

3.3.2. Noise decreases fry condition and increases mortality

Released fry were assessed on the day of release (0 days post-release, dpr) and 7, 14, 21, and 28 dpr. Since noise-exposed brooding females held their broods for longer, noise-exposed juveniles were inside the female’s mouth for ~2 days longer than control fish, so their dpr is shifted by this time frame (i.e. 28 dpr in noise-exposed
broods are ~2 days older than control juveniles). During the first 28 days post-release, juveniles exposed to noise during brooding differed in standard length compared to control juveniles (Figure 3.4A; 2-way RM ANOVA; treatment: $F_1 = 32.179, P < 0.001$; week: $F_4 = 668.015, P < 0.001$; treatment x week: $F_4 = 3.509, P = 0.014$). Because of the interaction between treatment (control vs noise) and time, post-hoc analyses were used to identify at which stages differences were present. Standard length did not differ at release ($P = 0.075$), but noise-exposed fish were significantly longer than controls at 7, 14, and 21 dpr ($P < 0.001$ for each), possibly due to being slightly older. However, by 28 dpr, juveniles had a similar standard length between treatments ($P = 0.381$). Body mass also differed with noise exposure and time (Figure 3.4B; 2-way RM ANOVA; treatment: $F_1 = 9.276, P = 0.010$; week: $F_4 = 287.885, P < 0.001$; treatment x week: $F_4 = 0.550, P = 0.700$). Overall, noise-exposed juveniles were smaller than control juveniles, and all fish were significantly larger than at the previous week ($P < 0.001$ for all comparisons). By calculating condition factor, which takes into account both body mass and standard length (see methods for formula), we found that control juveniles had higher condition factors than noise-exposed fish (Figure 3.4C; 2-way RM ANOVA; treatment: $F_1 = 61.544, P < 0.001$; week: $F_4 = 6.084, P < 0.011$; treatment x week: $F_4 = 3.547, P = 0.013$). Control juveniles had a higher condition factor at release ($P = 0.011$), and at 7 ($P < 0.001$), 14 ($P = 0.001$), and 21 dpr ($P < 0.001$), but by 28 dpr, fish had similar condition factors ($P = 0.550$). In addition, noise-exposed broods had higher mortality rates after release compared to control broods (Figure 3.4D; 2-way RM ANOVA; treatment: $F_1 = 17.921, P = 0.001$; week: $F_3 = 19.393, P < 0.001$; treatment x week: $F_3 = 6.909, P < 0.001$). Within control animals, there was no change in mortality
amongst the different weeks, however, in noise-exposed broods, animals had significantly higher mortality during the first week compared to weeks 2-4 ($P < 0.05$ for all). Up to 60% mortality during the first month was observed in noise-exposed broods, while control broods had at maximum 25% mortality during the first month (t-test; $t = -4.407, df = 12, P < 0.001$; Figure 3.4E, F).

Figure 3.4. Juveniles exposed to noise during development have slower growth rates and higher mortality. (A-B) Noise-exposed fish have greater standard length but lower body weight than control fish. (C) Noise-exposed fish have lower condition factors than control fish. (D-F) More noise-exposed fish per brood died during the first month post-release compared to silent-control broods, predominantly during the first week post-release. In D, values are cumulative of mortality that occurred during that week (i.e. value between days 0 and 7 represented the mortality during that week). Data was analyzed using 2-way RM ANOVAs with main effects and interaction p-values provided. Tukey’s post-hoc test was used to isolate specific differences as appropriate. In all graphs, filled circle with error bars represents mean ± s.d. In E and F individual data points are plotted as unfilled circles with mean ± s.d. plotted next to each group. Different capital letters represent differences within the noise group across time while differences in lowercase represent differences within the control group across time. * indicates differences between groups (i.e. between noise and control treatments). Different letters indicate statistical significance at $P < 0.05$. 

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3.3.3. Noise alters juvenile startle behaviors

When presented with an acoustic startle stimulus, all juvenile fish exhibited a startle response that resulted in freezing behaviors. We measured the time delay between the stimulus and when fish returned to normal swimming behaviors at 14 and 28 dpr. Juveniles exposed to noise during development took significantly longer to return to normal swimming compared to control fish at both time points (Figure 3.5A; 2-way RM ANOVA; treatment; $F_1 = 44.133$, $P < 0.001$; time; $F_1 = 0.002$, $P = 0.964$, treatment x week; $F_1 = 5.240$, $P = 0.043$). Shoaling behavior, or how close together fish swam in an open aquarium, was dependent on both sound condition during development as well as the time tested (14 vs 28 dpr) (Figure 3.5B; treatment; $F_1 = 20.788$, $P < 0.001$; week; $F_1 = 6.732$, $P = 0.027$; treatment x week; $F_1 = 1.161$, $P = 0.307$). Overall, noise-exposed juveniles swam closer together than control juveniles ($P = 0.001$), and fish swam closer together at 14 dpr than at 28 dpr ($P = 0.027$).

Figure 3.5. Shoaling and startle responses are affected by exposure to noise during development. (A) Fish exposed to noise during development take longer to return to normal swimming after an acoustic startle at both 14 and 28 days post-release. (B) Noise-exposed fish swim closer together compared to control fish at both 14 and 28 days post-release. Fish swim closer together at 2 weeks compared to 4 weeks, independent of sound treatment. Individual data points are plotted as unfilled circles with mean ± s.d. plotted next to each group. Different capital letters represent
differences within the noise group across time while differences in lowercase represent
differences within the control group across time. * indicates differences between groups
(i.e. between noise and control treatments) within each time point.

To assess if adult-typical behaviors/coloration were affected in juveniles after
they experienced noise exposure during the brooding period, we identified the first day
a fish from each brood was observed with adult-typical coloration or displaying an adult-
typical aggressive behavior. Control fish first displayed coloration ~30 dpr (29.778 ±
2.774 days) while noise-exposed broods did not develop colors until ~36 dpr (36.400 ±
3.364 days; t-test: t = -3.981, df = 12, P = 0.002; Figure 3.6A). Typically, only a single
(often the largest) fish was observed with yellow coloration and a faint eyestripe at this
stage. More complex coloration (vertical banding, fin spots, egg dummies) were not
present during the first 60 dpr in either group. A similar pattern was observed for the day
of first aggressive behavior. A single fish was observed chasing other fish from the
terracotta pot (i.e. territory), but no other adult-like aggressive behaviors were observed
during the first 60 dpr. This chasing behavior was first observed at ~31 dpr (30.667 ±
2.062 days) in control broods, but not until ~38 dpr (37.600 ± 4.393 days) in noise-
exposed broods (Figure 3.6B; t-test: t = -4.083, df = 12, P = 0.002). Control and noise-
exposed fish had similar adult-typical social behaviors and coloration later in life at ~4
months of age.

3.4. Discussion

Anthropogenic noise is now pervasive to almost all aquatic and terrestrial
environments. How animals cope with this increase in background noise is a recent
Figure 3.6. Exposure to noise during development delays the onset of adult-typical coloration and behaviors. (A) Noise-exposed juveniles first display adult-typical color (i.e. eyebar, yellow/blue body colors, fin spots) at a later age than control juveniles. (B) Similarly, juveniles exposed to noise during development begin to display territorial behaviors at a later date compared to control juveniles. dpr, days post-release. Individual data points are plotted as unfilled circles with mean ± s.d. plotted to the side of each group. Different letters indicate statistical significance at $P < 0.05$.

research focus because of its importance for species diversity, conservation management, and economic impacts. For fishes, the largest and most diverse group of vertebrates, underwater noise can have devastating effects on their growth, reproduction, and communication, with impacts observed both at individual and population levels (Board, 2005). Although previous work found that anthropogenic noise can impact growth and development to varying degrees (Bruintjes and Radford, 2014; Cox et al., 2012; Davidson et al., 2009; Nedelec et al., 2015; Wysocki et al., 2007), particularly in early life stages, no study has examined the impact of noise in mouthbrooding fishes. Mouthbrooding provides a unique situation because effects observed in juveniles could be direct (i.e. on developing juveniles themselves) or indirect (i.e. impacts on the brooding females that influence developmental conditions). We found that exposure to noise during mouthbrooding affected both the
mouthbrooding females themselves and the developing juveniles, potentially in interconnected ways.

Changes to parental care behaviors can have devastating effects on reproductive fitness of animals with potential consequences on species survival. In *A. burtoni*, a species completely reliant on mouthbrooding for reproductive success, exposure to noise during brooding resulted in dramatic changes to maternal care behaviors. Noise-exposed females were more likely to cannibalize or pre-maturely release underdeveloped larvae. In addition, females that did successfully carry and release a developed brood held onto their brood for significantly longer than control fish. Only one of the 12 noise-exposed fish fit the characteristics of a “typical” brooding period. Together, this resulted in > 90% of noise-exposed females with altered maternal care behaviors and only a 42% successful brooding rate. Of successful brooding females, noise-exposed females held onto their brood longer, but this could be considered advantageous since it allowed for further growth and protection to the developing juveniles. However, upon release, females were less likely to perform protective parental behaviors. Only 60% of noise-exposed brooders retrieved their brood when presented with a threat, compared to 100% of control females performing this common maternal care behavior. Perhaps there is a tradeoff between brooding time and post-release retrieval behaviors. Noise exposure shifted parental care behaviors from short brooding time with high post-release retrieval behaviors to a longer brooding time with diminished retrieval behaviors.

Anthropogenic noise affects parental care across taxa, with animals being less attentive during periods of noise (Algera et al., 2017a; Bruintjes and Radford, 2013;
Leonard and Horn, 2012; Maxwell et al., 2018; Naguib et al., 2013; Nedelec et al., 2017). For example, tree swallow parents visit their nest less frequently during periods of noise-playback compared to silent periods (Naguib et al., 2013). Male smallmouth bass (*Micropterus dolomieu*) guarding nests with egg-sac fry-stage offspring had decreased parental care behavior during noise (Maxwell et al., 2018), but effects were dependent on the stage of the offspring. Similarly, both cooperatively breeding cichlid *Neolamprologus pulcher* and spiny chromis damselfish (*Acanthochromis polyacanthus*) parents spent more time performing defensive behaviors and less time attending to their nest (Bruintjies and Radford, 2013; Nedelec et al., 2017). This change in time allocation means that nests/offspring are more prone to predation. Changes in parental care behaviors could be due to noise being a distractor or even changes in the circulating glucocorticoid levels induced by the noise. Cortisol negatively affects parental care behaviors and results in decreased nest success in smallmouth bass (Algera et al., 2017b). In tree swallows, glucocorticoids may be important for parents to strategically respond to offspring’s needs within different social and environmental contexts (Akçay et al., 2016). However, *A. burtoni* are unable to change their parental care strategies and are restricted to mouthbrooding. We do not know if our noise exposure protocol induced a change in cortisol levels, similar to that observed in several other fishes (Smith et al., 2004; Wysocki et al., 2006). However, a rise in cortisol due to the noise could explain the changes in maternal care that happen shortly after the noise exposure (i.e. spitting out underdeveloped fry, or cannibalism). Another, although also untested factor contributing to premature release and cannibalism could be noise-induced impacts on buoyancy. High intensity sounds can damage the swim bladder (Halvorsen
et al., 2012). Mouthbrooding A. burtoni females are already facing challenges to their buoyancy because of the mass of the developing brood in their buccal cavity. The female’s swim bladder is able to counteract this weight by shifting the proportion of gas between the anterior and posterior compartments (Butler et al., 2016). However, if exposure to noise is affecting gas exchange (either directly from the acoustic stimulus or stress-induced physiological changes) or damaging the swim bladder, this could affect their buoyancy and further add to their stress. Perhaps their premature release of underdeveloped fry is an attempt to correct these changes in buoyancy, raising interesting questions about motivational trade-offs between self-promoting and offspring-promoting behaviors during parental care. Nevertheless, our results, combined with past studies, indicate that parental care behaviors are extremely susceptible to noise-induced perturbations. As such, further research is needed in more species of varying parental care strategies to fully understand how changes in the environment, especially from anthropogenic noise, impact parental care and ultimately offspring survival and reproductive fitness.

Since developing juveniles are contained in the buccal cavity of brooding females, changes in the mother’s physiology and behavior could have direct consequences on juvenile development and/or noise could directly impact developing young themselves. We found that juveniles exposed to noise during development had lower condition factors. While these noise-exposed fish had similar body lengths, indicating similar growth rates, they had a lower body mass. We also noted that noise-exposed fish appeared to have very little fat or muscle mass, further suggesting that noise-exposed fish have a harder time putting on weight despite identical feeding
regimes and food availability. Interestingly, (Simpson et al., 2016b) found that exposure to anthropogenic noise increased the metabolic rate of developing Ambon damselfish (*Pomacentrus amboinensis*). In addition, higher cortisol levels associated with a stress response could cause decreased muscle mass (Crowley et al., 1996), possibly explaining lower body mass in noise-exposed fry after their release. In addition to changes in growth, noise-exposed juveniles had higher mortality during the first month, most commonly within the first week after release. While < 1% of control juveniles died during the first week after release, up to 50% of noise-exposed juveniles died during this same time. Mortality did stabilize slightly over the month, but total mortality of noise-exposed juveniles was 51.35% compared to 20.50% in control animals. This increased mortality during early life could be due to a lower condition and general poorer health. Nedelec et al. (2017) also found that juveniles exposed to noise during development had decreased survival likelihood but attributed this to higher predation. They did, however, observe that parental care-providing males performed less “glancing” behaviors, which transfers mucus to their offspring. This mucus contains proteins, hormones, immunoglobulins, ions, and microorganisms, which are important for offspring development and growth (Nedelec et al., 2017). Female *A. burtoni* are thought to provide mucus to their developing brood, which contains important components related to immunity, growth, and health (Keller et al., 2018). If the offspring receive less mucus from the parents, or if the mucus composition changes as a result of anthropogenic noise, this could decrease health of the offspring. Overall, decreased juvenile growth and increased mortality ultimately results in decreased reproductive fitness and can affect species persistence.
In addition to changes in physiology (i.e. growth), noise-exposed juveniles also had altered behaviors. Shoaling is a natural behavior observed in approximately half of all fishes when individual fish stay in close proximity to each other for social reasons (Shaw, 1978). In addition, shoaling may enhance protection from predators and increase foraging efficiency (Pitcher et al., 1982), and a fish’s motivation to shoal is affected by the quality (e.g. presence of parasites, nutritional condition) of their potential shoal-mates (Barber et al., 1998; Krause and Godin, 1996; Krause et al., 1999). We found that noise-exposed fish formed tighter shoals (i.e. swam closer together) than control fish. Shoals can provide protection (Hamilton, 1971; Seghers, 1974) and access to social information (Berdahl et al., 2013; Herbert-Read et al., 2015; Radakov, 1973), so fish swimming closer together could reflect a higher perceived threat or a greater need for information transfer between the fish. Interestingly, pile driving sounds affect both the structure and dynamics of shoals in juvenile seabass (Herbert-Read et al., 2017). Their shoals are less cohesive, less directionally oriented, and have a decreased correlation in speed and direction changes. This is attributed to noise likely interfering with the ability of fish to detect the mechanosensory signals from neighbors that is needed for shoaling and schooling behaviors (Faucher et al., 2010; Partridge and Pitcher, 1980). Since we saw the opposite effects, with noise-exposed animals being more cohesive, this is likely not due to changes in sensory input, but possibly reflective of increased stress. Our fish were placed in a novel environment without any shelter. Despite the acclimation period, these juveniles were likely stressed from the novel environment, with noise-exposed juveniles being more stressed than control broods.
This change in response to a novel environment and in shoaling behavior is likely to have detrimental effects on fish in naturally varying habitats.

In addition to shoaling, we used freezing time in response to an acoustic startle as a measure of stress. While all fish appeared to have a similar startle response to the acoustic stimulus (although this needs to be verified with high-speed video), the time it took for fish to return to normal behaviors after the startle differed with noise condition. In accordance with the changes observed with shoaling, noise-exposed juveniles took longer to return to normal swimming behaviors compared to the control juveniles. This longer “freeze” time traditionally indicates a higher stress level [for review see: (Koolhaas et al., 1999)]. While we did not rule out that noise exposure impaired the lateral line or auditory system, it is important to note that all fish responded to the acoustic stimulus. In addition, lateral line neuromast number and distribution was not affected by noise exposure (personal observations). Further, both lateral line and inner ear hair cells regenerate in fishes, so it is likely that if any damage to hair cells was present after the noise exposure, they had likely regenerated by the first behavioral test (~3 weeks after the exposure). As such, we propose that changes in freezing response following an acoustic startle and changes to shoaling behaviors are due to stress, and not deficits in hair cell-mediated communication.

Finally, we observed that noise-exposed juveniles had a delayed onset of adult typical coloration and behaviors. Typically, a single fish was observed to first display yellow coloration and/or an eyebar, which is characteristic of dominant males. This fish was observed chasing other fish from the shelter within several days of coloration onset. Together, this suggests the onset of adult-typical dominance behaviors. Juveniles
exposed to noise during development had a delayed onset compared to the controls. On average, the first sign of these adult-typical dominance behaviors was delayed by 7 days. This is particularly interesting as these noise-exposed juveniles are technically older because they were released ~2 days later than control fish. Despite the onset of coloration and territoriality at an early age, fish did not reach sexual maturity for several months, by which time there were no observable differences in coloration, behaviors, or reproductive success between the noise and control groups.

Taken together, our data indicate that noise exposure during development affects early-life (<1 month) behaviors and physiology. However, by ~1-month post release, noise-exposed fish were not different from juveniles that were not exposed to noise during development. By adulthood, these two groups of fish were indistinguishable based on condition factors and behavioral observations. Although more research is needed to test the exact physiological mechanisms leading to these changes, we propose that the observed early life effects are due to differences in stress physiology. While the noise may not directly impact the developing larvae, it is a stressor for the brooding female that could cause increases in her circulating steroids, including glucocorticoids. Developing larvae feed on the mucus and secretions from inside the brooding female’s mouth, which contains molecules important for immune function (Keller et al., 2018). Changes to the female’s stress physiology could cause changes to the mucus composition. If the fry are ingesting or exposed to higher levels of maternal glucocorticoids, this could affect their stress physiology and resilience (Hayward et al., 2004; Liu et al., 2001; Weinstock and immunity, 2005). We hypothesize that noise exposure induces a cortisol rise in brooding females which is passed on to her brood
through her mucus. This in turn makes their stress system more reactive, leading to transient changes in physiology and behavior during the first few weeks following the noise. We did not collect blood from the females because the amount needed for cortisol assays requires fish sacrifice, therefore not allowing us to obtain data on brood success and post-fry-release maternal behaviors. In addition, waterborne cortisol assays were not used because they would have added further stress to the experimental set-up. Future studies should examine circulating cortisol levels, as well as levels of cortisol and other important immune components in the brooding female’s buccal mucus during noise exposure.

3.5. Summary

Anthropogenic noise and increasing background sound levels are a prevalent problem in today’s world and are only projected to worsen in coming years. Territorial and site-attached animals living in noise-polluted areas are unlikely to leave, even in unfavorable conditions. We demonstrated here that a single exposure to noise during mouthbrooding had dramatic effects on maternal care behaviors. Over half of noise-exposed females failed to complete mouthbrooding successfully. Of those that did, their offspring were smaller and had higher mortality. Together, this resulted in significantly diminished reproductive fitness for the females. Since there is high diversity in parental care strategies among fishes and a broad range of acoustic communication and auditory capabilities, it is important to investigate noise-induced impacts on parental care and reproduction in a variety of species. Only after this has been done will we fully understand the detrimental impacts of human activities on fishes and be able to inform
policy makers on empirical-based ways to effectively alleviate this pervasive and worldwide problem.

3.6. References


Shaw, E. (1978). Schooling fishes: the school, a truly egalitarian form of organization in which all members of the group are alike in influence, offers substantial benefits to its participants. Am Sci. 66, 166-175.


4.1. Introduction

Anthropogenic noise is prevalent to almost all aquatic and terrestrial ecosystems. The rise in anthropogenic noise in recent decades has led to a large increase in the number of studies examining its impact on animal behavior, physiology, and communication. Underwater noise from shipping, pile driving, and oil exploration has caused ambient sound levels to increase over 30 dB in the frequency range that many fishes produce and detect acoustic stimuli (Board, 2005; Crovo et al., 2015; Purser and Radford, 2011; Radford et al., 2014; Scholik and Yan, 2001; Scholik and Yan, 2002a; Scholik and Yan, 2002b; Vasconcelos et al., 2007). With over 30,000 species of fishes important for ecological biodiversity to commercial food resources, it is imperative to understand how different species may cope with these increased noise levels.

One of the most prevalent impacts of anthropogenic noise on fishes is damage to sensory hair cells and shifts in hearing thresholds. Goldfish exposed to white noise for as little as 10 minutes had a 5 dB threshold increase while fish exposed to white noise for 24 hours suffered a 30 dB threshold shift that decreased their hearing sensitivity (Smith et al., 2004). Interestingly, for fish exposed to white noise for longer periods of time (up to 1 month) hearing thresholds stabilize at ~20 dB above baseline pre-exposure levels, and hearing sensitivity returned to baseline pre-exposure levels after 2 weeks in a quiet environment. This has been well studied because of the obvious effects of noise on hearing sensitivities and on the ability of animals to perceive both conspecific sounds and their environmental soundscape. However, few studies have
sought to understand what mechanisms may underlie hearing threshold shifts in fishes and if they have a protection mechanism against acoustic overstimulation. In mammals, for example, the cochlea has a proposed protective mechanism that resembles that of the hypothalamic-pituitary-adrenal (HPA) axis (Basappa et al., 2012). All components of the HPA axis, from the initiation of the axis with corticotropin releasing factor (CRF; (Graham and Vetter, 2011) to the final step of glucocorticoid receptors (Shimazaki et al., 2002; Terakado et al., 2011), are expressed in the organ of Corti. Because activity of the classic HPA axis can modulate homeostasis during times of stress, it was proposed that this inner ear HPA-like axis may mediate responses to acoustic stress, with focus on CRF signaling. In fact, mice lacking CRF-receptor 1 expression have a 20-30 dB deficit in auditory sensitivity but mice lacking CRF-receptor 2, have increased auditory sensitivity and are more prone to noise-induced hearing loss (Basappa et al., 2012; Graham et al., 2011; Graham et al., 2010; Graham and Vetter, 2011). While these studies demonstrate that the inner ear CRF signaling serves to protect the mammalian organ of Corti from noise-induced hearing loss, whether this or a similar protective mechanism also exists in other taxa remains unexplored.

Hair cells can be noise damaged by two primary modes. Physical damage to the hair bundles can result in severed tip links, and without functional tip links to selectively open mechanically-gated ion channels, hair cells cannot function properly. When stimulated, hair cells modulate their release of glutamate onto afferent nerves which then transmit signals to the brain (Ottersen et al., 1998). Overstimulation can also result in excess glutamate release into this synapse which can damage and even destroy the synapse. When glutamatergic AMPA receptors on the post-synaptic cell are continually
stimulated by glutamate, excess calcium can enter the cell and activate many different enzymatic pathways that lead to cellular damage. Interestingly, activation of the inner ear HPA-like axis in rodents promotes glutamine synthetase (\textit{glul}; enzyme that converts glutamate into glutamine) transcription in support cells of the rodent organ of corti (Basappa et al., 2012; Vardimon et al., 1999a) to protect against glutamate excitotoxicity.

Mammals are unable to regenerate hair cells, but fishes can grow entire functional hair bundles in ~2 weeks (Schuck and Smith, 2009). This suggests that it may be more important or adaptive for fish to quickly remove damaged hair bundles and replace them with new functional ones rather than repair damaged ones or employ protective mechanisms. In fact, in zebrafish, growth hormone and its related transcripts were upregulated following acoustic stress, and intraperitoneal injections of growth hormone increased hair cell proliferation (Schuck et al., 2011; Sun et al., 2011), suggesting that growth hormone may play a role in recovery from acoustic trauma.

Breeding and social state can influence sensory function across taxa. While it is well known that sex steroids can mediate acoustic communication in fishes (Maruska and Fernald, 2010a; Maruska and Sisneros, 2015; Sisneros et al., 2004), it remains unknown if social or reproductive state results in different levels of protection from noise-induced hearing loss. For example, if dominant and subordinate males have different expression levels of the CRF signaling pathway in the ear, will they have inherently different levels of protection from acoustic overstimulation? Since many fishes live in territorial systems or dominance hierarchies, or show reproductive state-dependent plasticity in sensory function, it is important to understand how hormonal
state impacts susceptibility to noise-induced threshold shifts, which has not been examined in any taxa.

Here we aimed to investigate if fish possess an inner ear CRF signaling system and determine how its expression is influenced by an animal’s physiological state. We found that all components of the CRF signaling system are expressed in the saccule of the social African cichlid fish, *Astatotilapia burtoni*. Expression was also sex and reproductive state dependent. After noise exposure, CRF signaling system components generally increased, but in a reproductive state-dependent manner. In contrast to higher expression of genes encoding ligands (i.e. *crfb, crfa, uts1*) and *crfr2* expression, *crfr1* expression decreased. Finally, we found that the noise exposure protocol used here, 3 hours of mixed pure tones spanning the hearing range of *A. burtoni*, resulted in damage to sensory hair cells. Overall, these data provide insight into the inner ear CRF signaling system as a potential conserved mechanism underlying noise-induced threshold shifts in vertebrates.

4.2. Materials and methods

4.2.1. Experimental animals

Adult *Astatotilapia burtoni* were laboratory-bred and maintained in community aquaria at conditions similar to their natural environments (pH = 7.6-8.0; 28-30 °C; 12 L:12 D diurnal cycle). Fish were fed cichlid flakes daily and brine shrimp thrice weekly. Community aquaria contained several halved terracotta pots to serve as territories for dominant males. All experiments were performed in accordance with the recommendations and guidelines stated in the National Institutes of Health (NIH) Guide
for the Care and Use of Laboratory Animals, 2011. All animal care and collection were approved by the Institutional Animal Care and Use Committee (IACUC) at Louisiana State University, Baton Rouge, LA.

Stable dominant and subordinate males were established by placing two size-matched territorial males into a tank containing a single spawning territory with 3 females. In this situation, one male becomes dominant over the other within an hour and resulted in stable social states that persisted for > 30 days. Only males with stable social states for >3 weeks were collected. Female *A. burtoni* breed year-round with a ~40 d cycle that is divided into three distinct phases: 1) gravid, reproductively receptive females identified by visibly distended abdomens due to large ova, 2) mouthbrooding, during which time the female provides sole parental care to the developing embryos by brooding them in their mouths for ~12-14 days, and 3) recovering, during which time vitellogenesis occurs and yolk deposits are replenished. Mouthbrooding females were collected 6-8 days after the onset of brooding and gravid females were identified based on a visibly swollen abdomen and presence of actively courting males. Upon dissection, reproductive state for all fish was verified by gonadosomatic index [=(gonad mass/body mass)*100]. A total of 95 fish were collected: 12 control dominant and subordinate males (= 24 fish); 15 control gravid and brooding females (= 30 fish); 8 fish per reproductive state in noise conditions (= 32 fish); 9 for *in situ* hybridization.

4.2.2. Sound exposure conditions

To examine how reproductive state and social status impacts expression of the inner ear CRF signaling system, saccule samples were collected from dominant males,
subordinate males, gravid females, and mouthbrooding females living in community aquaria. In community tanks, fish were exposed to low mechanical sounds associated with air pumps and filtration, as well as conspecific sounds (both intentional and unintentional). The ambient sound levels in community tanks typically did not exceed ~110 dB SPL re: 1 µPa. All fish for this control condition were collected from community tanks between 9-11 am.

To compare how noise exposure impacts expression of the inner ear CRF system, saccules were collected from noise-exposed dominant, subordinate, gravid, and brooding fish. The same sound conditions as described previously (Section 2.2.2, Section 3.2.2) were used. Fish were placed in a 38 L glass aquarium that was divided into two compartments by a blue opaque barrier. An underwater speaker (UW-30) was suspended vertically in the water of the back compartment so that no part of the speaker touched the tank. In the front compartment, a halved terracotta pot was positioned in the middle of the space. To create the “noisy” sound environment, a sound file was generated in Audacity v2.1.1 which consisted of pure tones ranging from 100 – 1500 Hz (the hearing range of A. burtoni). The order and duration (0.5 – 4 sec) of each pure tone frequency were randomized to prevent habituation. The sound file was approximately 5 min. in length but looped for 3 hours. The sound file was amplified (TOA, CA-160) and played through the underwater speaker so that it generated an ~140 dB (SPL re: 1 µPa) stimulus at the shelter.
4.2.3. Tissue collection and preparation

All fish were collected at the same time of day (9 am – 12 pm) to avoid any diurnal changes in gene expression. Animals were quickly netted from their aquaria (control: community tanks; noise: experimental tank) and measured for standard length (SL) and body mass (BM). Blood was collected from the caudal vein with heparinized 100µl capillary tubes, centrifuged at 8000 rpm for 10 minutes, and plasma was collected and stored at -80°C until analysis. Following sacrifice by rapid cervical transection, both sagitta (largest inner ear otolith thought to be the primary hearing organ in most teleost fishes) were quickly removed. The saccules (sensory epithelium surrounding each sagitta) were carefully dissected from the otolith, and the saccular nerve was trimmed so that only a small portion (~2 mm) remained proximal to the sensory macula. Saccules were then flash frozen and stored at -80°C until further analysis. RNA was extracted from homogenized saccular tissue using an RNeasy Plus Micro Kit (Qiagen) following the manufacturer’s protocol, reverse transcribed to cDNA, diluted 1:5 in nuclease-free water, and stored at -20°C until quantitative-RT PCR was performed.

For collection of saccular epithelia for in situ hybridization, fish were measured and euthanized as above. Brains were quickly exposed and the tissue surrounding the brain was loosened to allow adequate fixation. Heads were fixed overnight at 4°C in 4% paraformaldehyde (PFA) and rinsed in 1x phosphate-buffered saline (1x PBS; prepared from 10x stock; Fisher Scientific) for > 24 hours. Saccules were either stained whole (no further processing) or cryosectioned (Thermo Cryostar) at 20 µm following cryoprotection in 30% sucrose. Sectioned saccules were collected onto charged slides and allowed to dry flat at room temperature for 2 days before storage at -80°C.
4.2.4. Quantitative RT-PCR

To measure mRNA expression levels of the proposed inner ear CRF system, quantitative-RT PCR was performed on saccular epithelia of control and noise-exposed fish. Throughout this chapter, we use standard gene nomenclature. For fishes, gene names and symbols are italicized and protein names are capitalized. For other vertebrates, human conventions are used: gene symbols in all capitals and italicized, protein symbols in all capitals. When generally speaking about the system, all capitals are used. *Astatotilapia burtoni* have two forms of the corticotropin-releasing factor (CRF) gene [crfa, crfb; (Grone and Maruska, 2015)], a CRF-binding protein gene (crfbp), and two CRF receptor genes (crfr1, crfr2). In addition, *A. burtoni* have urotensin-1 (uts1), a CRF-like peptide that binds CRF-R2 with greater affinity than CRF itself. The glutamine synthetase (glul) enzyme, which converts glutamate into glutamine, is essential for protecting from glutamate excitotoxicity. We also measured expression of the gene encoding growth hormone (gh1), which is upregulated in the zebrafish saccule following noise exposure (Schuck et al., 2011). Gene specific primers were designed for crfa, uts1, glul, and gh1 based on available sequences, but all other primers (crfb, crfbp, crfr1, crfr2) have been used previously in *A. burtoni* (Carpenter et al., 2014; Chen and Fernald, 2008) (Table 4.1). Each primer pair had a single melt peak, and no primer set had amplification in no-RT controls.

qRT-PCR was performed on a CFX connect Real-Time system with reaction volumes of 20 µl in duplicate. Fluorescence thresholds for each sample were automatically measured (CFX manager, BioRad) and PCR Miner (Zhao and Fernald, 2005) was used to calculate reaction efficiencies and cycle thresholds using the
following equation: \[ \frac{1}{1+E_{\text{target}}}^{\text{CT}_{\text{target}}} \div \frac{1}{1+E_{\text{geomean}}}^{\text{CT}_{\text{geomean}}} \times 100 \], where E is the reaction efficiency and CT is the average cycle threshold of the duplicate wells. The relative amount of mRNA was then normalized to the geometric mean of two reference gene mRNA expression (18s and rpl32) and corrected for body size by dividing by fish standard length.

Table 4.1. Primer sequences used for qRT-PCR and ISH probe generation. * t3 transcription initiation sequence (AATTAACCCTCACTAAAGGG) was added to the reverse primer. 1(Zhao and Fernald, 2005), 2(Chen and Fernald, 2008), 3(Grone and Maruska, 2015).

<table>
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<td>GGAGCATTCGCTGGTATACG TCTTCCGGCTCACAAA</td>
<td>800</td>
</tr>
<tr>
<td>crfb2,3</td>
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<td>118</td>
<td>CCAGAATACTCCACACCTATTG ACCTCGTGGAGCCTT</td>
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<tr>
<td>uts1</td>
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<td>118</td>
<td>ACCTCGTGGAGCCTT</td>
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<tr>
<td>crfbp2</td>
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<td>crfr1,2</td>
<td>TTGCTAGGCTGCTCTGCA TTTGATTGCTGGCTCGGGAACGAGGCTCGGGCTCCGAGG</td>
<td>282</td>
<td>GGAGCATTCGCTGGTATACG TCTTCCGGCTCACAAA</td>
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</tr>
<tr>
<td>crfr2,2</td>
<td>TGCCACAGCAGGTAAGTGGAAACGAGGCTCGGGCTCCGAGG</td>
<td>113</td>
<td>CCAGGACTTCGCTGGTATACG TCTTCCGGCTCACAAA</td>
<td>800</td>
</tr>
<tr>
<td>glul</td>
<td>GTGGGTCTTTGTTTCTCTT TCCCTTCTCTTTCCTTTCCTCTCTC</td>
<td>145</td>
<td>GCAGCAGGAGTGGATACG AAGAAGACTA</td>
<td>721</td>
</tr>
<tr>
<td>gh1</td>
<td>TACCTGAGGCTGGCTAAAA GCAGGGCAAGGATATCAG</td>
<td>92</td>
<td>GCAGCAGGAGTGGATACG AAGAAGACTA</td>
<td>721</td>
</tr>
</tbody>
</table>

4.2.5. Preparation of DIG-labeled riboprobes and in situ hybridization

To localize each component of the CRF-signaling pathway in the saccular epithelia, we performed in situ hybridization using gene-specific riboprobes. Probe templates were amplified from whole brain (all probes but crfa) or eye (crfa) A. burtoni.
cDNA with gene-specific primers with the T3 polymerase recognition sequence appended to the reverse primer (Table 4.1). Eye cDNA was used to generate the crfa probe because crfa is not expressed in the brain of A. burtoni (Grone and Maruska, 2015). Digoxigenin (DIG)-labeled nucleotides were incorporated into the purified PCR product. Probes were purified, diluted in hybridization buffer, and stored at -20°C until use. During each step of probe generation, templates were verified to be a single band of the correct size using gel electrophoresis. Probe specificity was verified by running alternate brain sections with anti-sense (T3 sequence on reverse primer) and sense (T3 sequence on forward primer) probes. Sense-controls showed no staining for any candidate genes.

Whole saccules or slides of cryosectioned saccules were washed in RNase free 1x PBS (3 x 5 min), fixed in 4% PFA (20 min), washed in PBS (2 x 5 min), incubated in 10µg/ml proteinase K (10 min), rinsed in PBS (10 min), fixed in 4% PFA (15 min), washed again in PBS (2 x 5 min), rinsed in milliQ water (3 min), incubated for 10 min in 25% acetic anhydride in 0.1M triethanolamine-HCl (pH 8.0), and washed in PBS (5 min). After incubation in hybridization buffer at 60°C for 3 h, DIG-labeled gene specific probes were added and allowed to hybridize overnight at 55-60°C. The following morning, tissue was sequentially washed at 55°C in pre-warmed solutions of 2x standard sodium citrate (SSC):50% formamide (2 x 30 min), 1:1 SSC:maleate buffer (MABT; 2 x 15 min), and MABT (2 x 10 min). Tissue was then rinsed in MABT (2 x 10 min) at room temperature (RT), incubated in 2% BSA in MABT at RT for 3 h to block nonspecific binding, and incubated in anti-DIG antibody (1:5000 in blocking solution) at 4°C, flat overnight, in a humidified chamber. Following antibody incubation, tissue was
rinsed in MABT (3 x 30 min) at RT, washed in alkaline phosphatase buffer (2x5 min), and developed in SigmaFast FastRed solution at 37°C in the dark for 2-20 h depending on the probe. After development, tissue was rinsed in PBS (3 x 5 min), fixed in 4% PFA (10 min), and washed in 1x PBS (3 x 5 min). All washes after development were done in the dark. Wholemount-stained saccules were then mounted on slides, and all slides were coverslipped and counterstained with 4’, 6-diamidino-2-phenylindole (DAPI)-fluorogel. Slide edges were sealed with clear nail polish and stored flat at 4°C until imaging.

4.2.6. Imaging

To determine the location of CRF signaling system components in the saccule of *A. burtoni*, stained saccules were visualized on a Nikon Eclipse Ni microscope controlled by Nikon Elements software. Slides were viewed and photographed with a monochrome digital camera (Nikon DS QiMc) using appropriate wavelength filters. In sectioned saccules, rows of DAPI-labeled nuclei were used to determine the area of the sensory macula. To verify that this method allowed for consistent identification of saccular hair cells, several slides were stained for *vglut3*, the vesicular glutamate transporter found in all sensory hair cells. Location of staining was based on consensus from multiple animals of different sexes and on whole-mount stained and cryosectioned stained saccules.
4.2.7. Histological evaluation of saccular hair cells

To examine how noise exposure impacts sensory hair bundles of the auditory inner ear, saccules were stained with the f-actin stain phalloidin, which labels stereovilli. After noise exposure, fish were measured for standard length and body mass. Brains were quickly exposed, and the tissue surrounding the brain and otoliths loosened to allow fixation. Heads were fixed overnight at 4°C in 4% paraformaldehyde (PFA) and rinsed in 1x PBS for > 24 h. The entire sagitta/saccule complex was removed and a small slit was made in the lateral edge of the saccule to allow solution to penetrate the membranous sac. Whole mount phalloidin staining was used to visualize hair bundles of the inner ear. Tissue was washed for 5 minutes in 1x PBS before being gently transferred to 1% phalloidin (prepared in 1x PBS with 1% BSA and 0.1% triton-x) for 1 hour, washed in 1x PBS for 5 minutes, mounted onto a slide, and coverslipped and counterstained with DAPI fluorogel.

The number of intact and frayed hair bundles in the rostral, middle, and caudal portions of the saccule were quantified. To do this, slides were imaged at the highest magnification available using extended depth of field imaging. Z-stacked images were compressed and used for analysis. The rostral and caudal portions of the saccule could be identified based on the width of the macula since the sensory area is wider in the rostral portion of the saccule. For each part of the saccule, a 100 x 200 µm box was randomly placed in the center of the macula (away from the edges). The number of intact and frayed hair bundles within the ROI was quantified, and a ratio of intact to frayed bundles was computed.
4.2.8. Statistical analysis

All statistics were performed in SPSS 25 and SigmaPlot 12.3. To test for sex, reproductive state, and sound condition effects on gene expression in the saccule, body-size corrected relative expression levels were compared using a 3-way ANOVA followed by Tukey’s posthoc tests. Correlations were assessed using Pearson product moment tests when the data were normally distributed or Spearman rank tests when normality was not met. The percentage of frayed hair bundles was analyzed using a 2-way repeated measure ANOVA with sound condition (control, 1-hr noise, 3-hr noise) as the between-subject factor and location (rostral, middle, caudal) as the repeated within-subject factor. Tukey’s post-hoc testing was used to determine differences within factors and interactions. Data were first checked for outliers using Iglewicz and Hoaglin multiple outlier test with a Z of 3.5 (Iglewicz and Hoaglin, 1993). If data did not meet normality or equal variance, they were log, natural log, or square root transformed prior to analysis. Correlations and pairwise comparisons were not corrected for multiple comparisons. While Bonferroni and similar methods reduce the chance of type I errors, they also reduce statistical power and increase the chance of type II errors, potentially masking biologically relevant effects (Nakagawa, 2004). For these reasons, we chose not to use these conservative correction methods and instead present the observed effect size (e.g. r-values in correlations) and exact p values. Box plots are used throughout the text to represent data. The box extends to the furthest data points within the 25th/75th percentiles, and whiskers extend to the furthest data points within 1.5x the interquartile range. Outliers (beyond 1.5x the interquartile range) are designated by open circles and
are not reflective of statistical outliers. Data median is represented by a solid line and data mean by a filled circle.

4.3. Results

4.3.1. Localization of CRF signaling system in the saccule

*In situ* hybridization (ISH) was used to localize CRF signaling system components in the saccule. Since ISH denatures actin filaments, hair cells were identified by a single row of DAPI-labeled nuclei instead of the presence of phalloidin, which labels the hair bundles themselves. The sensory hair cell region could be reliably identified using this method in *vglut3* stained saccules (ubiquitously expressed in sensory hair cells; Figure 4.1). *Uts1* strongly stained in all sensory hair cells (Figure 4.1), *crfa* and *crfb* stained weakly in hair cells, but *crfr1* and *crfr2* stained primarily in non-sensory regions (i.e. outside the macula). *Crfr1* expression appeared in most areas outside of the sensory macula (support cells) while *crfr2* expression appeared weakly throughout support cells but more strongly in basal cells adjacent to hair cells. *Gh1* is known to be expressed only in sensory hair cells in the zebrafish saccule (Schuck et al., 2011; Sun et al., 2011). While we did not stain for glutamine synthetase, it is primarily expressed in support cells in the inner ear of other taxa (Eybalin et al., 1996; Nordang, 2000; Takumi et al., 1997).

4.3.2. Sex- and reproductive state-dependent expression

Using qRT-PCR, we measured expression of CRF signaling system components in the saccule of subordinate males, dominant males, brooding females, and gravid
Figure 4.1. Saccular location of CRF-signaling system components in A. burtoni. (A) Schematic representation of support cells (pink) surrounding the sensory macula and hair cells (yellow) and basal cells (gray) in the sensory macula. (B) In situ hybridization for vglut3 (red) showed distinct staining in hair cells, allowing for the identification of hair cells as a single row of cell nuclei (blue). (C) Expression of crfr1 (red) was strongest in support cells adjacent to the sensory macula. (D-E) crfb and uts1 expression (red) in hair cells of the sensory macula. (F) CRF system ligands (crfa, crfb, uts1) are located in the sensory macula (white) while CRF receptors are found in support, non-sensory cells (gray) or of basal cells (BC). gh1 and glul expression based on other studies. Scale bar = 25 µm.

females (Figure 4.2; see Table 4.2 for main effects; post-hoc P-values in text below). All components of the CRF-signaling system were amplified from A. burtoni saccular samples, and expression levels differed between reproductive states and sex under normal conditions. Females had higher expression levels of crfbp and both CRF receptors than males (crfbp: $P < 0.001$; crfr1: $P < 0.001$; crfr2: $P = 0.032$), but similar levels of all three ligands (crfa $P = 0.033$; crfb: $P = 0.407$; uts1: $P = 0.670$). Within females, brooding females had higher crfr1 expression than gravid females ($P < 0.001$), but similar levels of crfa ($P = 1.000$), crfb ($P = 0.999$), uts1 ($P = 0.996$), crfbp ($P = 0.989$), and crfr2 ($P = 0.998$). Within control males, dominant males had higher
expression of crfa than subordinate males \((P = 0.012)\), but similar levels of crfb \((P = 1.00)\), uts1 \((P = 0.998)\), crfbp \((P = 1.00)\), crfr1 \((P = 0.999)\), and crfr2 \((P = 1.00)\).

After noise exposure, females had higher expression of crfa and crfb than males \((crfa: P = 0.033; crfb: P = 0.001)\), but no differences in expression of uts1 \((P = 0.670)\), crfbp \((P = 0.280)\), crfr1 \((P = 0.379)\), or crfr2 \((P = 0.099)\). Within females, brooding females had higher expression of all measured genes \((crfa: P < 0.001; crfb: P = 0.006; uts1: P = 0.041, crfbp: P = 0.002, crfr2: P < 0.001)\) except crfr1 compared to gravid females \((P = 0.729)\). Noise-exposed dominant males had higher expression of uts1 in the saccule than subordinate males \((P = 0.007)\), but similar expression levels of both crf genes \((crfb: P = 0.977; crfa: P = 0.764)\), crfbp \((P = 0.663)\), and both crf receptors \((crfr1: P = 0.785; crfr2: P = 0.381)\).

Under normal conditions, there were no sex or reproductive state differences in either glutamine synthetase \((glul)\) or growth hormone \((gh1)\) expression. However, after noise exposure, females had higher glul expression than males \((P = 0.002)\). Within females, brooding females had higher glul expression than gravid females \((P < 0.001)\). Growth hormone in noise-exposed saccules was similar across sex and reproductive state \((P > 0.05)\).

4.3.3. Noise-induced changes to CRF signaling system expression in the saccule

When comparing expression levels between control and noise-exposed fish, sound condition significantly affected expression levels of crfa, crfb, uts1, crfbp, crfr1, crfr2, and glul, but not gh1 \((P = 0.012)\), but similar levels of crfb \((P = 1.00)\), uts1 \((P = 0.998)\), crfbp \((P = 1.00)\), crfr1 \((P = 0.999)\), and crfr2 \((P = 1.00)\).

After noise exposure, females had higher expression of crfa and crfb than males \((crfa: P = 0.033; crfb: P = 0.001)\), but no differences in expression of uts1 \((P = 0.670)\), crfbp \((P = 0.280)\), crfr1 \((P = 0.379)\), or crfr2 \((P = 0.099)\). Within females, brooding females had higher expression of all measured genes \((crfa: P < 0.001; crfb: P = 0.006; uts1: P = 0.041, crfbp: P = 0.002, crfr2: P < 0.001)\) except crfr1 compared to gravid females \((P = 0.729)\). Noise-exposed dominant males had higher expression of uts1 in the saccule than subordinate males \((P = 0.007)\), but similar expression levels of both crf genes \((crfb: P = 0.977; crfa: P = 0.764)\), crfbp \((P = 0.663)\), and both crf receptors \((crfr1: P = 0.785; crfr2: P = 0.381)\).

Under normal conditions, there were no sex or reproductive state differences in either glutamine synthetase \((glul)\) or growth hormone \((gh1)\) expression. However, after noise exposure, females had higher glul expression than males \((P = 0.002)\). Within females, brooding females had higher glul expression than gravid females \((P < 0.001)\). Growth hormone in noise-exposed saccules was similar across sex and reproductive state \((P > 0.05)\).
Figure 4.2. All components of the CRF-signaling system are expressed in the _A. burtoni_ saccule. Relative mRNA levels of the CRF-signaling system in the saccules from mouthbrooding females (yellow), gravid females (green), dominant males (red), and subordinate males (blue). Expression of the CRF signaling system in saccular epithelia in control (A) and noise-exposed (B) animals is dependent on sex and reproductive status. Expression of _glul_ and _gh1_ in control animals (C) is not dependent on sex or reproductive state, but _glul_ expression is varies with female reproductive state in noise-exposed fish (D). Gene expression is relative to expression of 2 reference genes and corrected for body size (see methods for details). Different lowercase and uppercase letters represent statistical differences within males and females, respectively. * represents differences between males and females. See methods for box plot description.
Table 4.2. Expression of CRF-signaling molecules in the saccule varies with sex, reproductive state, and noise exposure. Results from three-way ANOVA on saccular gene expression (body size corrected) with sex, reproductive state, and sound condition as fixed factors and interactions (sex X sound condition; reproductive state X sound condition). Degrees of freedom (df) are for sex, state, sound, and residuals.

<table>
<thead>
<tr>
<th>gene</th>
<th>df</th>
<th>sex</th>
<th>state</th>
<th>sound</th>
<th>sex X sound</th>
<th>state X sound</th>
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<td>crfb</td>
<td>1,3,1,65</td>
<td>10.969</td>
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<td>6.185</td>
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<td>37.523</td>
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<td>10.000</td>
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<td>0.670</td>
<td>4.171</td>
<td>0.009</td>
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<tr>
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<td>&lt;0.001</td>
<td>5.121</td>
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<td>&lt;0.001</td>
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<tr>
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<td>&lt;0.001</td>
<td>47.640</td>
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<td>&lt;0.001</td>
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<td>0.390</td>
<td>0.534</td>
<td>0.896</td>
<td>0.448</td>
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Saccules were higher than control saccules, except for crfr1, in which a sex-specific decrease was observed. For all measured genes except gh1, there was a significant interaction between sound condition (noise vs control) and sex (crfb, crfr1, crfbp, glul) or reproductive state (crfb, crfa, uts1, crfbp, crf1, crfr2, glul). Expression of uts1, crfr2, and glul was higher in noise-exposed brooding females (uts1: \( P = 0.002 \); crfr2: \( P < 0.001 \); glul: \( P < 0.001 \)) and dominant males (uts1: \( P < 0.001 \); crfr2: \( P = 0.002 \); glul: \( P = 0.002 \)) compared to control fish, but there was no difference between noise-exposed and control gravid females (uts1: \( P = 0.967 \); crfr2: \( P = 0.085 \); glul: \( P = 0.347 \)) or subordinate males (uts1: \( P = 0.498 \); crfr2: \( P = 0.114 \); glul: \( P = 0.309 \)). Crfa expression was higher in noise-exposed brooding females (\( P < 0.001 \)), dominant males (\( P = 0.003 \)), and subordinate males (\( P = 0.008 \)), but not in gravid females (\( P = 0.884 \)) compared to control animals. Crfb expression was higher in noise exposed brooding females (\( P < 0.001 \)), gravid females (\( P = 0.031 \)), and dominant males (\( P = 0.039 \)), but not subordinate males (\( P = 0.094 \)). Effects of noise on crfr1 expression were sex-specific. In
both gravid and brooding females, \textit{crfr1} expression was lower in noise-exposed females compared to control females (gravid: \( P = 0.030 \); brooding: \( P < 0.001 \)). In males, however, \textit{crfr1} expression did not differ between noise-exposed dominant and subordinate males compared to control males (dominant: \( P = 0.085 \); subordinate: \( P = 0.442 \)). Expression of \textit{crfbp} also had an interesting reproductive state-dependent effect. Noise-exposed brooding females and dominant males had higher levels of \textit{crfbp} expression than control fish (brooding: \( P = 0.045 \); dominant: \( P = 0.004 \)). In contrast, noise-exposed gravid females had lower \textit{crfbp} expression than control gravids (\( P = 0.014 \)), and there was no difference in subordinate individuals (\( P = 0.064 \)). There was no effect of sound condition on \textit{gh1} expression. Overall, noise exposure impacted expression in brooding females and dominant males but had little to no impact in gravid females or subordinate males, indicating sex and reproductive state-dependent effects of noise exposure on expression of CRF signaling system components in the saccule.

To better understand how components of the CRF signaling system relate to each other and \textit{glul} and \textit{gh1} expression, we used Pearson correlations within each sound condition and reproductive state (Figure 4.4; Table 4.3). In general, \textit{crfb} or \textit{uts1} expression positively correlated with \textit{glul} expression in control animals. There were only 3 to 7 significant correlations (out of 28 possible) in control animals, but noise-exposed animals had \(~18\) significant correlations. Interestingly, \textit{crfr1} expression did not correlate with any other gene in noise-exposed saccules, but \textit{crfr2} expression positively significantly correlated with \textit{crfb}, \textit{crfa}, \textit{uts1}, \textit{crfbp}, \textit{glul}, and \textit{gh1}. A stark outlier to the high number of correlations in noise-exposed animals is subordinate males. While most CRF signaling system components significantly correlated with each other in brooding
Figure 4.3. Noise-induced changes in gene expression are reproductive state-dependent. N = 12-15 control fish per group; 8 noise fish per group. Different letters
represent statistically significant differences between control and noise-exposed animals within a reproductive state. Differences across reproductive state or sex are plotted in Figure 4.3 for ease of visualization. See methods for box plot description.

females, gravid females, and dominant males, only two correlations were present in noise-exposed subordinate males. However, they did retain the significant correlation between uts1 and crfbp with both glul and gh1, suggesting a possible connection between the proposed CRF signaling system and those molecules in noise-exposed animals. In addition, glul and gh1 significantly correlated together in all noise-exposed saccules but no control saccules, indicating that their correlated rise in the expression may be tied to acoustic trauma.

Figure 4.4. Correlation of CRF-signaling system expression varies with noise exposure and reproductive state. Only a few correlations are observed in control (top/right portion of each box) saccules, but components of the CRF-signaling system strongly correlate with each other, glul, and gh1 expression in noise-exposed animals (bottom/left portion of each box). Color represents correlation coefficient. * represent statistically significant correlations (stats in Tables 4.3 and 4.4).
Table 4.3. Correlations of saccular gene expression in control animals. Pearson correlations of CRF signaling system components expression with each other and glutamine synthetase (glul) and growth hormone (gh1).

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<tr>
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<th>crfa</th>
<th>uts1</th>
<th>crfbp</th>
<th>crfr1</th>
<th>crfr2</th>
<th>glul</th>
<th>gh1</th>
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<td></td>
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<td>-0.300</td>
<td>-0.059</td>
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Table 4.4. Correlations of saccular gene expression in noise-exposed animals. Pearson correlations of CRF signaling system components expression with each other and glutamine synthetase (glul) and growth hormone (gh1).

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### 4.3.4. Noise exposure leads to damaged hair cells

While we know that noise exposure impacts hearing thresholds in gravid females (Chapter 2), the primary receivers of intentional sound production in this species, we wanted to assess if noise exposure resulted in damaged hair bundles or if threshold shifts were due solely to changes in synaptic signaling between the hair cell and afferent nerve (i.e. via the CRF signaling system). To investigate hair cell integrity, we stained saccules with phalloidin and quantified the number of intact and frayed hair bundles in the rostral, middle, and caudal portion of the saccular macula (Figure 4.5). Both one and three hours of noise exposure resulted in a higher percentage of damaged cells (i.e. lower intact:damage ratio; 1-h: $P < 0.001$; 3-h: $P < 0.001$), with three hours resulting in more damage than one hour ($P = 0.005$). In control animals, the ratio of intact to damaged hair bundles was similar in the rostral, middle, and caudal saccule ($P = 0.245$). However, within the noise-exposed groups, the rostral region had significantly more damage than the middle and caudal regions of the saccule ($P < 0.05$ for all), but the middle and caudal regions were similar (1-h: $P = 0.122$; 3-h: $P = 0.835$).
Figure 4.5. Noise exposure results in damaged sensory hair bundles. Representative photomicrographs of phalloidin staining (green) in the saccule of *A. burtoni* at various magnifications. The box in A represents the area quantified for the posterior portion and is shown at higher magnification in B. The number of intact (C) and disrupted (D) hair bundles were quantified at high magnification. (E) Both one and three hours resulted in more frayed hair bundles (i.e. lower ratio of intact to frayed). The rostral saccule (green) had more damage the middle (blue) and caudal (red) portions of the saccule in both noise-exposed groups. Arrows indicated individual hair bundles in C-D. Scale bars: A = 100 µm, B = 25 µm, C-D = 10 µm.

4.4. Discussion

Anthropogenic noise has increased ambient sound levels in both terrestrial and aquatic ecosystems. High intensity sounds, like pile driving, are often short in nature, but can have devastating effects on overall health (Casper et al., 2013). While low intensity sounds often have lower, sub-lethal effects, their impacts are often longer-lasting (Board, 2005). Animals must find ways to cope with this pervasive and constant noise. The auditory system has a unique way of protecting itself from acoustic overstimulation. In mammals, components of the HPA axis are expressed in the
cochlea, have important functions in regulating hearing thresholds, and serve in a protective mechanism against noise-induced excitotoxicity (Basappa et al., 2012; Graham et al., 2011). Here, we tested if components of the CRF signaling system are expressed in the main inner ear organ of fish, investigated how their expression varied with sex and reproductive state, and examined how exposure to noise impacted expression levels. First, we found that all components of the CRF-signaling system were expressed in the A. burtoni saccule in a sex and reproductive state dependent manner. While ligand expression (i.e. *crfb*, *crfa*, and *uts1*) did not vary with sex or reproductive state, receptors and *crrbp* expression were reproductive state-dependent. After noise exposure, expression of all three CRF ligands, *crfr2*, *crfbp*, and glutamine synthetase increased, but *crfr1* expression decreased. There was no change to growth hormone expression, and its expression did not differ between sexes or reproductive states. Interestingly, noise-induced changes in gene expression varied with sex and reproductive state, such that an animal’s response to pervasive anthropogenic noise is likely dependent on their physiological condition.

In the mammalian cochlea, the two CRF receptors have opposing functions. CRF-R1 activation leads to increased hearing sensitivities (Graham and Vetter, 2011), while activation of CRF-R2 leads to decreased hearing sensitivities (Basappa et al., 2010; Graham et al., 2010). Knocking out either receptor significantly decreases or increases thresholds, respectively. We found that components of the CRF signaling system were expressed in a sex- and reproductive state-dependent manner. Levels of *crfbp* and both CRF receptors were higher in females than in males. Further, *crfr1* expression was higher in brooding females than gravid females. As such, *crfr1*
expression does not appear to convey an overall increase in hearing capabilities in fishes since gravid females have better hearing capabilities (at 200-300 Hz) compared to brooding females (Maruska et al., 2012). Crfr1 expression is tied to glutamine synthetase expression (Graham and Vetter, 2011), such that more CRF signaling through CRF-R1 promotes glutamine synthetase expression. Under normal conditions, this allows for faster glutamate recycling so that there is no glutamate deficiency in the presynaptic hair cells. We found that females had lower crfr1 expression after noise exposure compared to control females. Based on what is known in mammals, we expected glul expression to also decrease due to the glucocorticoid response element present in a glul promoter region, which would reduce glutamate-glutamine turnover. However, we found that glul expression actually increased following exposure to noise. This increase in glul expression could increase glutamate turnover, lowering available glutamate present at the synapse, and help to protect against glutamate excitotoxicity of the post-synaptic afferent nerve cell. In mammals, crfr1 and glutamine synthetase expression are highly related to systemic glucocorticoids (Vardimon et al., 1999b), and the role of local CRF signaling or glucocorticoid regulation of crfr1 and glul are not well understood.

In contrast, CRF-R2 has a known protective role in cellular stress-response mechanisms in the mammalian cochlea (Basappa et al., 2010; Graham et al., 2011). Signaling through CRF-R2 has important antioxidative roles, and through its interaction with connexin expression in support cells, is able to blunt effects of cellular oxidative stress in the cochlea (Graham et al., 2010). CRF-R2 also has an important role in regulating neurotransmission and/or afferent signal transduction by modulating subunit
composition of glutamate receptors in the afferent nerve. Glutamate is released from inner hair cells and binds AMPA glutamate receptors located on afferent nerves (Altschuler et al., 1989; Eybalin et al., 2004; Eybalin et al., 1996). Noise exposure results in decreased GluR2/3 subunits and increased GluR4 subunits and homomers (Graham et al., 2010). GluR4 homomers are more sensitive to desensitization (Pang et al., 2008), and thus, results in post-synaptic attenuation in response to constant noise. CRF-R2 in mammals appears to regulate the addition of GluR4, such that crfr2 KO mice have a 70% higher rise in GluR4 following noise exposure (Graham et al., 2010). We found that, in contrast to crfr1 expression decreasing, crfr2 expression increased following noise exposure in A. burtoni. Although the exact role of GluR4-comprised AMPARs is not fully known, in mammals it is proposed that the increase in GluR4 homomers following noise suppresses synaptic transduction because of their rapid desensitization (Graham et al., 2011). A decrease in crfr2 expression, resulting in decreased signaling through CRF-R2, could result in more GluR4 subunits. A higher proportion of GluR4 homomer receptors could lead to faster desensitization during prolonged noise exposure in A. burtoni. This mechanism is partially responsible for temporary threshold shifts observed following noise in mammals and could also explain noise-induced threshold shifts in fishes. Further studies using CRF agonists and receptor antagonists are needed to investigate the functional roles of crfr1 and crfr2 in hearing capabilities of fishes.

To understand how the CRF-signaling system functioned as a whole, we correlated expression of ligands, receptors, and the CRF binding protein with each other. We found that CRF receptor ligands (crfa, crfb, and uts1) correlated significantly
with \textit{crfr2} expression in noise-exposed saccules, while \textit{crfr1} did not correlate with any component of the CRF signaling system. This is consistent with what is proposed in mammals. CRF-R1 signaling is more related to a systemic stress response, but CRF-R2 functions in a local protective CRF-signaling mechanism within the inner ear. When looking at the system as a whole, it is also important to consider how CRF binding protein expression changes with expression of ligands. CRF binding protein prevents CRF ligands from binding to both CRF receptors in teleosts (Manuel et al., 2014), however, the complete function and regulation of CRFBP is still unknown. Interestingly, \textit{crfbp} expression increased following noise exposure in brooding females, and both dominant and subordinate males. In gravid females, however, noise-exposed females had lower \textit{crfbp} expression than control females. Even though ligand expression increased more in brooding females and males, the rise in binding protein may negate the increase in ligands. However, gravid females also had an increase in \textit{crfb} and \textit{uts1} expression following noise. This, coupled with lower \textit{crfbp} expression, may result in increased signaling through the CRF receptors and potentially an enhanced physiological-state dependent response to continuous noise.

While the proposed inner ear CRF-signaling system can provide protective effects \textit{during} noise exposure by reducing neurotransmission, it does not provide a protective effect on the hair bundles themselves. Overstimulation and excessively loud sounds can damage sensory hair bundles (Schuck and Smith, 2009; Smith et al., 2006). Under normal conditions, fish are able to continually regenerate hair cells. The number of saccular hair cells correlates with body size and appears to plateau with age when size is constant (Higgs et al., 2002; Popper and Hoxter, 1984). In some fishes, the
number of hair cells varies with reproductive state and season (Coffin et al., 2012) such that reproductively ready fishes during the breeding season have more saccular hair cells than non-breeding animals and those in non-breeding seasons. As such, fishes are constantly removing and replacing hair cells. Exposure to white noise or anthropogenic sounds results in damaged hair cells, fewer intact hair cells, and pits or scars in the saccular macula in goldfish, bass, and cichlids (Casper et al., 2013; Smith et al., 2006). Apoptosis is seen immediately after noise, and other cell death markers also increase in the days following noise exposure (Schuck et al., 2011).

After removal of damaged hair cells, new ones must be produced. Growth hormone has emerged as one of the candidates regulating hair cell regeneration in fishes (Schuck and Smith, 2009; Schuck et al., 2011; Sun et al., 2011). Gh1 transcripts are upregulated 64-fold when measured 2 to 4 days after noise exposure in the zebrafish saccule (Schuck et al., 2011). Injecting fish with growth hormone increases cell proliferation, but its affects vary between inner ear structures. The utricle had a higher level of GH-induced cell proliferation than the saccule, and within the saccule, the rostral portion was more sensitive than the caudal portion. The only gene measured in the present study that was not affected by sound exposure was growth hormone. However, this is likely because our samples were collected immediately after noise exposure. While gh1 expression was trending towards higher levels in noise exposed animals, it was not statistically different, but significance may have been seen if measured in fish allowed to recover from the noise for a day or two.

In comparison with the role of the CRF system, much less is known about further down-stream HPA-axis components (e.g. ACTH, corticosteroids, corticosteroid
receptors) in the inner ear and how/if they have a role in protecting against noise-induced hearing loss. Systemic steroid administration is a clinical treatment for idiopathic sudden sensorineural hearing loss, tinnitus, Menière’s disease, and various autoimmune diseases with associated hearing loss. Despite oral treatment with steroids improving hearing in up to 50% of patients (Canlon et al., 2007), the mechanisms of how glucocorticoids modulate hearing is not well understood. Further, dissociating local versus systemic corticosteroid actions is difficult. Glucocorticoid response elements are found abundantly in the genome, indicating that glucocorticoids can have major impacts on transcription of various genes. Some proposed roles of glucocorticoids involve increased biosynthesis of glutathione, decreased cytokines, and changes to expression of apoptotic genes likely to protect against free radical damage [reviewed in (Basappa et al., 2012)]. In the guinea pig vestibular system, glucocorticoid administration results in hyperexcitability of cells (Shimogori et al., 1999), and in the rat cochlea, glucocorticoid receptor (GR) activation can up- and down-regulate various proteins (Yao et al., 1995). Acoustic stress resulted in decreased GR expression in the organ of Corti and other cochlear tissues in rats (Rarey et al., 1995). While our focus was on the CRF signaling system in the saccular epithelia, previously Maruska & Fernald (2010) found that glucocorticoid and mineralocorticoid receptors are also expressed in saccular epithelia in a sex and reproductive state-dependent manner. Females had higher expression of all corticosteroid receptors than males. Subordinate males had higher expression of all three glucocorticoid receptors (gr1, gr2a, gr2b) and mineralocorticoid receptor (mr) compared to dominant males. Gr1 expression was highest in saccules from brooding females and lowest in gravid females. Recovering females had higher gr2a and gr2b
expression than both gravid and brooding females. Gravid females also had lower
eexpression of mr compared to brooding and recovering females. Interestingly, there was
little evidence that circulating cortisol levels correlated with corticosteroid receptor
expression in the A. burtonii saccule. While the exact roles of corticosteroids in the inner
ear are not fully understood, it is clear that corticosteroid signaling via glucocorticoid
receptors, much like CRF signaling, mediates hearing capabilities, varies with acoustic
trauma, and can have important consequences for modulating cellular stress.

4.5. Summary

Due to changes in acoustic soundscapes, animals must be able to cope with low
levels of continuous or intermittent noise. For fishes, increases in shipping have caused
underwater noise levels to rise significantly in the frequency range that most species are
able to detect. Exposure to anthropogenic noise is linked to changes in hearing
capabilities. Temporary threshold shifts could be due to either decreased synaptic
signaling and/or damaged hair cells. This is the first study to examine if the proposed
inner ear CRF signaling system exists in fishes. While we did not test the functionality of
this system, our results lay the ground work for future studies using receptor agonists
and antagonists. If the inner ear CRF system functions similar to that of mammals, our
results suggest that upregulation of crfr2 and down regulation of crfr1 work together to
modulate neurotransmission at the hair cell synapse. This decrease in synaptic activity
could lead to temporary threshold shifts that allow fish to filter out continuous
background noise. These threshold shifts could, however, also result in decreased
capabilities of detecting relevant social or environmental stimuli, an important phenomenon that remains to be tested.

4.6. References


CHAPTER 5. CONCLUSIONS

Human-generated noise pollution is of global concern. Increases in shipping, sonar use, pile driving, and more have all contributed to a rise in ambient underwater sound levels. Unfortunately, continuous low intensity sounds, like shipping noise, are pervasive in shallow-shore environments where many social species live (Board, 2005). Shipping noise also corresponds to the frequency range that many fishes produce and detect acoustic stimuli (Crovo et al., 2015; Purser and Radford, 2011; Radford et al., 2014; Scholik and Yan, 2001; Scholik and Yan, 2002a; Scholik and Yan, 2002b; Vasconcelos et al., 2007). Because acoustic communication is used primarily in reproductive contexts across fishes, disruption of communication can have negative consequences on reproductive success. In addition to changes to reproductive interactions, noise can impair territorial behaviors, predation risks due to distraction, and development. Together, these all negatively impact species persistence.

In chapter 2, I examined the impact of noise on social behaviors and communication. I provide one of the most comprehensive studies on noise-induced changes to social communication by looking at impacts on both the sender and receiver. Communication theory posits that it is the sender’s responsibility to maximize the likelihood of signal reception by modifying signal attributes (i.e. location, timing, type) to align with environmental conditions (Bradbury and Vehrencamp, 1998). Noise has the potential to alter the sender’s production of the signal, mask the signal itself (if acoustic), or change the receiver’s physiology. While I was unable to test exactly how the signal was masked by the noise file, I found that courtship sounds were undetectable when overlaid with the noise file, suggesting that the signal is masked, or
at least distorted by the sound. I also found that males change their behaviors during male-male territorial interactions and spent more time with their eyebar displayed, suggesting a potential increase in visual signaling. During reproductive interactions, I found that males change the location of the courtship behaviors. Instead of producing courtship quivers (and associated sounds) immediately next to gravid females, males produced these behaviors inside their spawning shelter. This change in location decreases the likelihood of the female detecting it. To further complicate acoustic communication, I found that noise-exposed gravid females had lower hearing sensitivity at 200 and 300 Hz, a major component of male courtship sounds. Together, these data indicate that noise has the potential to impact all three components of social communication: signal production, signal reception, and the signal itself. Subtle changes to social behaviors and communication, while not dramatic effects, are important to evaluating sublethal impacts of noise on reproductive success and species survival.

In chapter 3, I provide the first evidence that anthropogenic noise impairs mouthbrooding and maternal care behaviors. Parental care is a crucial life history stage where animals are more susceptible to stressful conditions (Parent et al., 1995). I found that exposure to noise during mouthbrooding decreased brooding success. Females were more likely to cannibalize their brood or prematurely release under-developed fry. When females did retain their brood, they held on to them for longer, possibly as an extra measure of protection. Under normal conditions, females will provide post-release maternal care behaviors by taking their young into their mouth when threatened. However, females that were exposed to noise during brooding did not perform these typical maternal care behaviors. These changes in female behavior resulted in
drastically reduced brooding success, demonstrating that even a single exposure can have detrimental impacts lasting beyond just the timeframe of exposure. Also in chapter 3, I found that a single exposure to loud noise during development can have detrimental impacts extending into the first month post-release. Noise-exposed juveniles had lower growth rates and higher mortality than control individuals. They also had altered shoaling and startle behavior and a delayed onset of adult-typical social behaviors. Together, these data indicate that a single exposure to anthropogenic noise at a critical life history stage or developmental time point can have long-lasting impacts.

Finally, in chapter 4, I tested the hypothesis that fish possess an inner ear CRF signaling system. In mammals, the HPA-axis equivalent signaling system in the cochlea provides protection from acoustic overstimulation (Basappa et al., 2012; Graham et al., 2011). I found that all components of the CRF signaling system are expressed in the main inner ear organ of a fish. Further, I demonstrated that its expression is both sex and reproductive state-dependent. Following noise, crfr1 expression was downregulated while crfr2 expression was upregulated. Together, this potentially helps to reduce synaptic transmission from hair cells to the afferent nerve. This is the first evidence of the CRF system in the saccule of any fish. While functional manipulation of this system is still needed, my results suggest that this may be a conserved mechanism that functions during exposure to prolonged noise to downregulate synaptic transmission and protect against noise-induced hearing loss.
5.1. Remaining questions and future directions

While the results of my dissertation provide valuable insights on how exposure to noise impacts fish behavior, physiology, communication, and development, it also proposes several interesting questions for future research.

Terrestrial and aquatic animals are facing changes in their acoustic environment due to increases in anthropogenic noise. Since anthropogenic noise is specific to the auditory system (and possibly mechanosensory system in fishes), several studies have tested for noise-induced effects on acoustic signal production. For example, anurans and birds exposed to anthropogenic noise modify the volume and frequency at which they call (Díaz et al., 2011; Fuller et al., 2007; Grafe et al., 2012; Nemeth et al., 2013; Ríos-Chelén et al., 2015). Only recently have studies begun to examine how disruption of one sensory channel may influence signaling in another modality. In cuttlefish, a cephalopod that uses complex visual signaling but does not produce auditory signals, exposure to anthropogenic noise increases the amount of visual signals produced (Kunc et al., 2014). Similarly, in the Bornean rock frog, exposure to anthropogenic sounds causes modifications to both auditory and visual signaling (Grafe et al., 2012).

Although it was recently found that fish will modify sound production during playback of human-generated sounds (Holt and Johnston, 2014; van Oosterom et al., 2016), no study to date has investigated the impact of anthropogenic noise on multimodal signaling in fish. Further, no study has investigated the impact of anthropogenic noise on signaling across all sensory modalities in any taxa.

In chapter 2, I provided preliminary evidence that exposure to noise might have cross-modal impacts on social communication. By increasing the time with their eyebar
displayed, males have increased visual signaling of their dominance status. However, it remains unknown if this is intentional visual signaling or a by-product of increased stress. Future studies are needed to directly test signaling in multiple sensory channels at the same time. Both male and female A. burtoni have contextual urine release (Field and Maruska, 2017; Maruska and Fernald, 2012), such that they increase urination directed towards potential reproductive mates and during aggressive interactions. Males also increase their use of visual displays when exposed to ready-to-reproduce females (Butler et al., 2019). When exposed to noise, do males increase their signaling in undisturbed sensory channels to compensate for disrupted acoustic communication? Future studies should examine chemosensory signaling (via urine release), visual signaling (number/type of displays; brightness/richness of coloration), and auditory signaling (proportion of courtship quivers associated with grunts) to understand how noise impacts multimodal sensory communication.

With changes to social behaviors, it is also important to understand what is happening in the brain that may underlie these changes. By collecting brains after social interactions, immediate early genes can be measured to determine regions of the brain that are differentially activated during different sound conditions [for example, see (Butler and Maruska, 2016)]. If coupled with behavior analysis and/or examination of multimodal signaling, future studies could assess how neural processing of socially-relevant information is impacted by anthropogenic noise. Further, transcriptomic approaches could be used to examine noise-induced changes in gene transcripts within specific brain regions (and the saccule itself) that could also be correlated with behavioral outcomes.
In chapter 3, I found that noise significantly impacted mouthbrooding. Females were more likely to cannibalize their brood or release immature juveniles. While I was able to document these behavioral changes, I was not able to isolate a potential mechanism or cause. One likely mechanism relates to a noise-induced stress response in brooding females. Loud noises cortisol levels in several species of fishes (Smith et al., 2004; Wysocki et al., 2006). I did not measure cortisol levels because handling females for blood collection during brooding is not conducive to future behavior experiments. However, as an ongoing project, I collected brains and blood from a separate group of control and noise-exposed brooding females following the same protocols described in Chapter 3. I will measure circulating cortisol levels from these females using an ELISA. Brains collected from control and noise-exposed brooding females were also macrodissected, and RNA samples from the telencephalon, hypothalamus, and midbrain were sent for transcriptome analysis. By determining up- and down-regulated genes in noise-exposed brains, this future work will provide important information on potential mechanisms underlying the dramatic changes in mouthbrooding behavior.

Also in chapter 3, I found that a single exposure to noise during critical developmental time points has long-term impacts on juvenile development and behavior, but the mechanisms of these prolonged impacts remains unknown. One possible explanation could be changes to developing juveniles stress physiology. Future studies are needed to examine baseline and stress-induced cortisol levels in noise-exposed individuals. Because of the size of juveniles, collecting enough serum for hormone analysis will be impossible. Instead, pooling together fish for water-borne
hormone assays may be effective. In addition, cross-fostering experiments would provide relevant and useful information on parent-offspring interactions. Are the observed changes to juvenile growth, mortality, and behavior due to direct effects of noise exposure on developing juveniles or indirectly from the female? Since developing juveniles feed off of buccal mucus, alteration to the composition of the mom’s mucus, like increased glucocorticoids or immune system components, could have potential impacts on development and programming of juvenile stress and immune systems. By swapping broods between noise-exposed and control brooding females, cross fostering experiments may begin to tease apart parent-offspring interactions. We collected heads from developing juveniles immediately after noise exposure and have sent them for transcriptome analysis. RNA-seq analysis will allow us to determine what genes are up- and down-regulated following noise exposure and provide insight into potential mechanism underlying the long-term deficits associated with noise-exposure during development. Further, I am measuring cortisol levels from yolk-sacs of control and noise-exposed animals. It would also be interesting to examine noise-induced epigenetic effects (e.g. methylation) on mothers and their fry, which could have implications for transgenerational impacts on species survival.

In chapter 4, I found that components of the CRF signaling system vary with noise exposure, such that decreased signaling through R1 and increased signaling through R2 may work together to decrease synaptic function and reduce overstimulation from constant noise. Future studies are needed to functionally test the role of the CRF system in noise exposure. To do this, CRF agonists or receptor antagonists should be used in control and noise-exposed animals. Agonizing or antagonizing each CRF
receptor in control animals will help to determine if the two receptors have a conserved function in hearing capabilities across taxa. By manipulating the CRF signaling system, exposing animals to noise, and testing for noise-induced hearing loss, it can be determined if the CRF system provides protection during prolonged noise exposure. Since males and females have different hearing capabilities, and thresholds are modulated by reproductive state within a sex, it is possible that noise-induced threshold shifts may be dependent on physiological condition. Similarly, because components of the CRF signaling system are expressed and modulated by noise in a sex and reproductive state dependent manner, future studies are needed to examine if there is a sex/state dependent protection from prolonged noise. For example, gravid females have increased levels of ligands but lower levels of CRF-binding protein following noise. Does this lead to increased signaling through CRF receptors, and therefore, confer increased modulation of neurotransmission resulting in greater threshold shifts? It would also be interesting to examine this CRF signaling system in a broader evolutionary context across all vertebrate taxa to understand the selective pressures shaping this protective hearing mechanism.

Because noise exposure also results in hair cell damage, it would also be important to examine mechanisms involved in both apoptosis and regeneration of hair cells in the saccule. The removal and replacement of hair cells may also differ with fish reproductive state and sex, and therefore these mechanisms following noise exposure may also differ. Fishes likely have multiple mechanisms to ensure they can hear sounds in their environment, and understanding the relative importance of protection versus addition of new hair cells is important.
While my research focused on the impacts of a single exposure of anthropogenic noise on behavior, communication, and development, future studies are still needed on the impacts of long-term exposure to anthropogenic sounds. Do animals eventually acclimate to the noise? Alternatively, are there long-term impacts on social behaviors and communication? How does continued exposure, similar to that in a shipping lane or harbor, affect stress physiology? Since glucocorticoids can negatively impact reproductive physiology, future studies should also examine the long-term impacts of noise on factors like circulating sex steroid levels, sperm maturation and motility in males, female reproductive cycle length, and reproductive rates.

5.2. References


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