Plasticity within the Auditory Systems of Fishes

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Plasticity within the Auditory Systems of Fishes

by

Melissa Macksoud

A Thesis
Submitted to the Faculty of Graduate Studies
through the Department of Biological Sciences
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Windsor, Ontario, Canada

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Plasticity within the Auditory Systems of Fishes

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April 24, 2019
DECLARATION OF ORIGINALITY

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ABSTRACT

Fishes inhabit incredibly cacophonous environments and experience functional, morphological, and transcriptional auditory system plasticity in reproductive state-dependent and auditory experiential contexts. In contrast to the comprehensive study of acoustic overexposure and functional reproductive condition-dependent plasticity within the auditory periphery, the mechanisms underlying acoustic experience-mediated central nervous system plasticity in fishes are generally poorly characterized. Recent research has highlighted neurochemical and transcriptional flexibility within the central nervous systems of fishes in response to prolonged exposure to music. However, the contributions of the acoustic characteristics of musical stimulation to central nervous system plasticity remain unclear. To evaluate the contributions of sound stimulus frequency to brain plasticity, I employed a targeted transcriptional analysis of neuroplasticity-associated genes within the brain of zebrafish (*Danio rerio*) exposed to 100 Hz and 800 Hz continuous pure tones at a sound pressure level of 140 dB (re 1 μPa) for 1-week intervals across a 4-week period. The transcription of genes involved in mediating connective plasticity fluctuated as a function of duration and frequency of sound exposure, while cellular proliferation did not show variation with sound treatment; suggesting prolonged tonal stimulation may facilitate connective plasticity within the zebrafish brain. These results provide evidence of central nervous system plasticity in response to pure tone exposure and implicate sound-induced behaviour and multisensory inputs in the mediation of sound-induced transcriptional flexibility within the zebrafish brain. Collectively, this thesis highlights the complexity of auditory system plasticity and emphasizes the value of investigating acoustic experience-mediated nervous system plasticity beyond the auditory periphery in fishes.
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TABLE OF CONTENTS

DECLARATION OF ORIGINALITY ................................................................. iii
ABSTRACT .................................................................................................... iv
ACKNOWLEDGEMENTS .............................................................................. v
LIST OF TABLES ........................................................................................... ix
LIST OF FIGURES ........................................................................................ x

CHAPTER 1 PLASTICITY WITHIN THE AUDITORY SYSTEMS OF FISHES 1

  1.1. Audition in Fishes .................................................................................... 2
  1.2. Neuromodulators as Facilitators of Reproductive State-Dependent Auditory
      Plasticity in Fishes ................................................................................... 3
      1.2.1. Hormones Mediate Reproductive State-Dependent Plasticity of the Auditory
             Circuit in Fishes .................................................................................. 6
      1.2.2. Morphological and Gene Expression Plasticity within the Auditory Systems is
             Associated with Reproductive Condition ........................................... 12
      1.2.3. Catecholaminergic Innervation Varies as a Function of Reproductive
             Condition ............................................................................................ 15
  1.3. Auditory Plasticity Driven by Altered Experience ...................................... 20
      1.3.1. Environmental Noise-Induced Hearing Loss and Peripheral Damage .......... 21
      1.3.2. Auditory Stimulation as a Means of Environmental Enrichment ............... 27
  1.4. Concluding Thoughts ............................................................................... 34

References ..................................................................................................... 36
Tables ............................................................................................................ 61

CHAPTER 2 EVIDENCE FOR SOUND-INDUCED NEUROPLASTICITY:
LONG-TERM PASSIVE EXPOSURE TO MODERATE-LEVEL SOUNDS
INFLUENCES GENE EXPRESSION WITHIN THE ZEBRAFISH BRAIN ...... 65

  2.1. Introduction .......................................................................................... 65
  2.2. Methods .................................................................................................. 68
      2.2.1. Animal Care ..................................................................................... 68
      2.2.2. Auditory Stimuli ............................................................................. 68
      2.2.3. Sound Exposure Protocol ................................................................. 69
      2.2.4. Tissue Preparation for Histological Analysis ...................................... 69
      2.2.5. Immunohistochemistry, Image Acquisition, and Analysis ................. 69
      2.2.6. Selection of Candidate Genes ............................................................ 70
      2.2.7. RNA Extraction and cDNA Synthesis .............................................. 71
# Table of Contents

2.2.8. Quantitative Real-Time Polymerase Chain Reaction ........................................ 71  
2.2.9. Primer Amplification Efficiency and Quantification Cycle Determination ........ 71  
2.2.10. Evaluation of Endogenous Control Genes ...................................................... 72  
2.2.11. Normalization and Calculation of Log-Transformed Fold Change ............... 72  
2.2.12. Behavioural Analysis ..................................................................................... 73  
2.2.13. Statistical Analyses ....................................................................................... 73  
2.3. Results .................................................................................................................. 74  
2.3.1. Brain-Derived Neurotrophic Factor Transcription ....................................... 74  
2.3.2. Neuronal Differentiation Factor 1 Gene Transcription .................................... 75  
2.3.3. Atonal Basic Helix-Loop-Helix Transcription Factor 1a Transcription .......... 76  
2.3.4. Proliferating Cell Nuclear Antigen Gene Expression ..................................... 76  
2.3.5. Swimming Behaviour in Response to Long-Term Pure Tone Exposure ........ 78  
2.4. Discussion ........................................................................................................... 78  
2.4.1. Long-Term Sound Exposure Induces Duration-Dependent Transcriptional  
       Flexibility in a Gene-Specific Manner ................................................................. 79  
2.4.2. Transcriptional Effects of Long-Term Sound Exposure are Frequency-  
       Dependent .............................................................................................................. 83  
2.4.3. Frequency-Specific Behavioural Plasticity Poses Implications for Central  
       Nervous System Transcription ............................................................................ 85  
2.4.4. Study Limitations ............................................................................................ 88  
2.5. Study Conclusions and Implications .................................................................. 90  
References .................................................................................................................. 92  
Tables .......................................................................................................................... 104  
Figures ......................................................................................................................... 105  

CHAPTER 3 CONCLUSIONS AND RECOMMENDATIONS ........................................ 112  
3.1 Summary ............................................................................................................. 112  
3.2 Future Directions ................................................................................................. 114  
References .................................................................................................................. 116  

APPENDIX A: GRAPHS ............................................................................................... 118  
APPENDIX B: PROTOCOLS ....................................................................................... 124  
VITA AUCTORIS ....................................................................................................... 125
LIST OF TABLES

Table 1.1. Summary of auditory (musical) environmental influences on the dopaminergic, serotonergic, and noradrenergic brain neurochemistry of fishes....61

Table 2.1. Summary of gene primer sequences employed and empirically estimated polymerase chain reaction efficiencies of real-time quantitative reverse-transcription polymerase chain reaction assays……………………104
LIST OF FIGURES

Figure 2.1. Wave forms of acoustic stimuli employed for zebrafish (*Danio rerio*) auditory environment treatments.................................................................105

Figure 2.2. Log$_2$-transformed fold change in the zebrafish (*Danio rerio*) whole brain expression of neuroplasticity-associated genes in response to prolonged acoustic pure tone exposure.........................................................106

Figure 2.3. Number of proliferating cell nuclear antigen (PCNA)-immunopositive cells within hindbrain auditory and lateral line system nuclei of zebrafish (*Danio rerio*) following long-term pure tone exposure................................................108

Figure 2.4. Time-course of locomotor activity of zebrafish (*Danio rerio*) in response to long-term exposure to tonal sound stimulation.............................110

Figure 2.5. Brightfield images anti-proliferating cell nuclear antigen immunohistochemical staining within the descending octaval nucleus of the zebrafish auditory hindbrain.................................................................111

Figure A.1. Normalized transcript abundance of candidate neuroplasticity genes within the zebrafish (*Danio rerio*) brain in response to long-term acoustic pure tone exposure.................................................................118

Figure A.2. Relative mRNA expression levels of targeted neuroplasticity-associated genes within the zebrafish (*Danio rerio*) whole brain following prolonged acoustic tonal exposure. ........................................120

Figure A.3. Fold change of neuroplasticity-associated gene transcription within the zebrafish (*Danio rerio*) brain following long-term acoustic pure tone exposure..122
CHAPTER 1
PLASTICITY WITHIN THE AUDITORY SYSTEMS OF FISHES

Traditionally viewed as hard-wired (Vasama & Mäkelä, 1995), the vertebrate auditory system is capable of a large degree of plasticity governed by both endogenous and environmental factors (Forlano et al., 2016; Petersen & Hurley, 2017; Hurley & Kalcounis-Rueppell, 2018; Irvine, 2018). For example, endogenous estrogenic neuromodulation mediates the detection and processing of salient acoustic stimuli across the reproductive cycles of vertebrates (Caras, 2013) while environmental noise-induced trauma can disrupt the central topographic map of sound frequency within the rat brain (e.g. Masri et al., 2018). Auditory experience plays a role in facilitating central auditory system plasticity (Kandler et al., 2009); however, gross plasticity in early sensory areas as a consequence of auditory experience is limited to a critical period in mammals (Zhang et al., 2001; Chang & Merzenich, 2003; de Villers-Sidani et al., 2007; but see Patton et al., 2019). In contrast, the central and peripheral nervous systems of fishes are characterized by an unparalleled level of neural plasticity among vertebrates (Kaslin et al., 2007) with no defined critical period (Näslund et al., 2012). Considering the plastic nature of the tetrapod auditory system, it is not surprising that it is of ichthyic origin (Fay & Popper, 2000).

Fish inhabit incredibly dynamic cacophonous environments in which flexibility of responsiveness to the sonic environment is often crucial for the survival and reproductive success of individuals. Detecting, filtering, and processing auditory information is often necessary to detect predators (Remage-Healey et al., 2006) and prey (Holt & Johnston, 2011), evaluate potential mates (Amorim et al., 2015), identify potential competitors (Remage-Healey & Bass, 2005), and maintain group cohesion (Van Oosterom et al., 2016). While fish possess a relatively simple nervous system, its plastic nature enables flexibility in sensation, perception, and auditory-evoked behavioural responses in an internal state- (Forlano et al., 2016; Thompson & Mangiamele, 2018) and external context- (e.g. Remage-Healey & Bass, 2005; Remage-Healey et al., 2006) dependent manner.

This review focuses on the drivers and mechanistic repertoire underlying the reproductive state- and experiential-dependent modification of the auditory system. In
reviewing recent studies of plasticity within the auditory systems of fishes in response to
endogenous and exogenous drivers, I summarize the extensively studied system-level
alterations in fish audition — reproductive condition-dependent neuromodulation and
acoustic trauma-induced plasticity — and the underpinning mechanisms facilitating this
plasticity in function. Additionally, I draw attention to the scarcely researched
experientially-driven auditory system plasticity within adulthood through the examination
of the genetic and molecular consequences of auditory experience. With this review, I
intend to provide a synthesis of the current findings regarding functional plasticity of the
fish auditory system and identify areas for future research to encourage the integrative
investigation of auditory system plasticity in fishes.

1.1. Audition in Fishes
Fishes experience their profoundly acoustic environment through the
mechanosensory acoustico-lateralis system organs: the inner ear and lateral line system
(Higgs & Radford, 2013; Higgs & Radford, 2016). In aquatic environments, sound
consists of both a particle motion and pressure component (Rogers & Cox, 1988), of
which the former is detected through integrative auditory and lateral line system inputs
(Higgs & Radford, 2013; Higgs & Radford, 2016). The otolithic end organs of the inner
ear; the saccule, utricle, and lagena, predominately facilitate the detection of particle
motion in audition (Popper & Lu, 2000). Despite the auditory potential of the utricle and
lagena in many species, auditory reception in fishes is disproportionately credited to the
saccule (Popper & Lu, 2000; Ladich & Schulz-Mirbach, 2016). Within each inner ear end
organ resides sensory epithelia overlain with bundles of hair cells which, upon otolith-
mediated deflection, transduce neural activity and convey auditory information to the
afferent nerve fibers of the VIII cranial nerve (Ladich & Schulz-Mirbach, 2016). It is
important to note, however, that the contributions of the mechanosensory lateral line are
difficult to distinguish from that of the auditory modality during low frequency (< 400
Hz) sound detection (Higgs & Radford, 2013; Higgs & Radford, 2016). As in the
auditory system, the lateral line system end organs — superficial and canal neuromasts
— are arrayed with sensory hair cells which are exposed to the external environment and
receptive to particle motion as a result of hydrodynamic flow (Metcalf et al., 1987;
Engelmann et al., 2000; Raible & Kruse, 2000). In addition to the reception of particle
displacement, some fishes have evolved specialized mechanisms which mediate sound pressure detection (Popper & Fay, 2011). Otophysan fishes are characterized by the affiliation of a Weberian apparatus with the inner ear, permitting the detection of sound pressure stimuli and effectively extending the hearing and spectral sensitivity of these teleosts (Fay & Popper, 1974; Ladich & Wysocki, 2003; Ladich & Schulz-Mirbach, 2016). The Weberian apparatus, a series of modified vertebrae (Watson, 1939; Adams, 1940; Fink & Fink, 1981; Ladich & Popper, 2004; Dahdul et al., 2010), mechanically couple the vibratory wall of an air-filled cavity (e.g. respiratory structures or swim bladder) with the inner ear and facilitate the transduction of sound pressure to particle motion (Popper & Hawkins, 2018).

Despite the multisensory integration involved in the sensation and perception of acoustic stimuli in fishes (Kasurak et al., 2012; Higgs & Radford, 2013; Estramil et al., 2014), I focus my discussion principally on audition and auditory system plasticity and draw upon investigations of the lateral line system which exploit the mechanosensory physiological similarities between these systems to provide further support for the conclusions of the present review and stimulate future research.

1.2. Neuromodulators as Facilitators of Reproductive State-Dependent Auditory Plasticity in Fishes

Neuromodulators play a critical role in facilitating context-specificity of communication across the reproductive cycle in acoustically communicating vertebrates (Remage-Healey & Bass, 2006; Lynch, 2017; Petersen & Hurley, 2017). Through the promotion of neural circuit functional flexibility, neuromodulators contribute to the broad repertoire of actions performed by seemingly fixed sensory neural networks (Hoke & Pitts, 2012; Petersen & Hurley, 2017; Nienborg & Jacob, 2018). Networks mediating reproductive behaviours are the recipients of significant neuromodulatory inputs, promoting reproductive condition-specific behaviours (Remage-Healey & Bass, 2006; Lynch, 2017; Petersen & Hurley, 2017). Studies of the neural substrate underlying acoustic signal processing in reproductive contexts have revealed the plastic nature of vertebrate auditory system across the reproductive cycle is attributed to steroid hormone and monoaminergic neuromodulation within the auditory circuit (Caras, 2013; Forlano et al., 2016; Maney & Rodriguez-Saltos, 2016; Schofield & Hurley, 2018). During periods
of reproductive receptivity, neuromodulation appropriates the auditory circuitry to optimize the detection and processing of salient acoustic signals (Arch & Narins, 2009; Caras, 2013; Forlano et al., 2015; Caras & Remage-Healey, 2016; Forlano et al., 2016; Forlano & Sisneros, 2016). In fishes, this differential engagement of the auditory system in response to acoustic signaling across reproductive contexts as a result of neuromodulation is a prominently researched facet of auditory plasticity (reviewed in Forlano et al., 2015; Forlano et al., 2016; Forlano & Sisneros, 2016).

The auditory system of acoustically communicating fishes provides a powerful model system for analyzing how neuromodulators facilitate auditory system plasticity and how altered circuit dynamics relate to reproductive behaviour (Forlano et al., 2015; Forlano et al., 2016; Forlano & Sisneros, 2016). First, despite species-specific and within-species reproductive morphotype differences in mating strategies, the presentation of acoustically-evoked responses to male courtship acoustic signals is temporally dynamic in many species and mirrors reproductive cyclicity [seasonal: plainfin midshipman (*Porichthys notatus*) (Arora, 1948) and round goby (*Neogobius melanostomus*) (MacInnis & Corkum, 2000); monthly: *A. burtoni* (*Astatotilapia burtoni*) (Kidd et al., 2013)]. Within these acoustic mating systems, reproductive females exhibit a great propensity for robust and direct phonotaxis in response to the playback of spectral components and recordings of male conspecific acoustic mating signals (Ibara et al., 1983; McKibben & Bass, 1998; Rollo et al., 2007; Zeddies et al., 2010; Kasurak et al., 2012; Maruska et al., 2012; Isabella-Valenzi & Higgs, 2013). Second, the temporal cyclicity of neuromodulator expression parallels reproductive condition, auditory-evoked behaviour, and auditory sensitivity (Sisneros et al., 2004b; Rohmann & Bass, 2011; Maruska et al., 2012; Zeyl et al., 2013). Finally, the teleost auditory system is plastically receptive to neuromodulation through the abundant and timely expression of enzymes necessary for neuromodulator synthesis and associated receptors (Forlano & Bass, 2005a; Forlano & Bass; 2005b; Maruska & Fernald, 2010). Taken together, the temporal dynamics of reproductive acoustic behaviour and the parallel fluctuations in neuromodulators with the easily tractable physiological and morphological attributes of fish auditory systems make them an ideal model to better understand how
neuromodulation can facilitate plasticity of responses in the auditory system and ensuing behaviour.

The study of neuromodulator-associated auditory function in fishes often takes a sensory neuroethological approach due to readily observable inter- and intrasexual dimorphisms and seasonality of communication that occurs naturally within many acoustic mating systems of fishes. Three common models for acoustic work in this context are the *A. burtoni*, an African cichlid (Maruska et al., 2012), round goby (Rollo et al., 2007; Rollo et al., 2008; Isabella-Valenzi & Higgs, 2013; Zeyl et al., 2013) and especially, the plainfin midshipman (e.g. Sisneros and Bass, 2003; Sisneros et al., 2004a; Sisneros, 2009; Rohmann & Bass, 2011; Forlano et al., 2016). The mating systems of these species include intrasexual male dimorphisms in reproductive behavioural phenotypes (Fernald & Hirata, 1977; Brantley & Bass, 1994; Marentette et al., 2009). Plainfin midshipman males exist in two alternative reproductive morphs: “sneaker” type II males steal fertilizations from “singing” type I males while they are engaged in acoustic courtship with females at their nests (Brantley & Bass, 1994). In contrast, male *A. burtoni* adopt reversible dominant territorial and subordinate non-territorial mating tactics in which only dominant males typically court females (Fernald & Hirata, 1977). While morphological and physiological intrasexual dimorphisms suggest the round goby may also exhibit alternative male reproduction tactics (Marentette et al., 2009), behavioural evidence of these alternative male reproductive strategies is lacking (Kornis et al., 2012) and neurophysiological studies of the round goby to date have characterized males and females as monomorphically reproductive and non-reproductive (Laframboise & Zielinski, 2011; Zeyl et al., 2013). While generally beyond the scope of the present review, the study of inter-sexual dimorphism in auditory-evoked behaviour and physiology helps to contextualize the relevance of functional changes in audition and behaviour facilitated by neuromodulation across the reproductive cycle.

The majority of knowledge gained since the discovery of seasonal auditory plasticity in the plainfin midshipman (Sisneros & Bass, 2003) has centered on the hormonal neuromodulation of the fish auditory circuit (Forlano et al., 2016). Further delineating the underpinning mechanisms of auditory system plasticity in acoustically communicating species across the reproductive cycle, more recent studies have
implicated catecholamines (Forlano & Sisneros, 2016), gonadotrophin-releasing hormone (Maruska & Tricas, 2011), and morphological mechanisms (e.g. Rohmann et al., 2013) in the mediation of reproductive-condition dependent audition. Below, I review these plastic physiological and morphological mechanisms as facilitators of the plastic detection and perception of attractive, competitive, and species-specific acoustic signals across the reproductive cycle in fishes.

1.2.1. Hormones Mediate Reproductive State-Dependent Plasticity of the Auditory Circuit in Fishes

The fish auditory system is the recipient of extensive hormonal influences (Forlano et al., 2015; Forlano et al., 2016) which are implicated in the complex regulation of sensory processing in reproductive contexts (Thompson & Mangiamele, 2018). In addition to the systemic hormonal influences on the brain mediated by the hypothalamic-pituitary-gonadal axis, hormones can be synthesized within the substrates of sensory systems and exert their neuromodulatory effects locally (Abe & Oka, 2011; Diotel et al., 2018). De novo steroidogenesis, the enzymatic conversion of cholesterol to steroid hormones, has been demonstrated within the periphery and brain of fishes (Diotel et al., 2018). Estrogen and androgen stimulation of nuclear- and membrane-bound steroid hormone-specific receptors promotes transcriptional activity and intracellular signaling cascades, respectively (Thomas, 2012; Nelson & Habibi, 2013), thus modulating the neural responses of sensory system substrates (Thompson & Mangiamele, 2018). Similarly, extrahypothalamic neurons producing gonadotrophin releasing hormone II and gonadotrophin releasing hormone III within the brain have been implicated in the modulation of reproductive behaviour through neuromodulatory effects on sensory circuits (Abe & Oka, 2011). Thus, neurohormones originate systemically and from locally produced steroidogenic precursors allowing rapid manipulation of neural circuit functionality (Abe & Oka, 2011; Diotel et al., 2018).

In acoustic mating systems, hormones can rapidly tune auditory circuits to acoustic mating stimuli and regulate behavioural responses (Caras, 2013; Forlano et al., 2016; Wilczynski & Burmeister, 2016; Burmeister, 2017; Vahaba & Remage-Healey, 2018). These reproductive condition-dependent auditory-evoked behavioural responses have since been attributed to differential auditory function, a neurophysiological correlate
of circulating sex steroids and their actions upon the auditory system in acoustically communicating fishes (Forlano et al., 2015).

The discovery of enhanced auditory saccular afferent responsivity in female plainfin midshipman during the breeding season to tones which constitute the conspecific male mating call by Sisneros and Bass (2003) and subsequent hormonal induction of this trait in non-reproductive females (Sisneros et al., 2004a) represented a major breakthrough in the understanding of neuromodulatory mechanisms underlying the seasonal-dependency of phonotaxis in fishes and largely inspired the field of steroid hormone-mediated plasticity of audition in fishes. Electrophysiological recordings revealed reproductive females exhibit elevated precision of phase-locking, spike rates, and synchronized units within auditory afferent fibers of the VIII nerve in response to the higher harmonic components of male vocalizations than their non-reproductive counterparts (Sisneros and Bass, 2003). Similarly, saccular-evoked potentials measured in response to these spectral components revealed females possess improved hearing sensitivity for the higher harmonic frequencies of the call while reproductively receptive (Sisneros, 2009; Rohmann & Bass, 2011). The fluctuations of auditory sensitivity within the peripheral nervous system of females closely mirrors reproductive condition and associated changes in the levels of systemic steroid hormones (Rohmann & Bass, 2011). Female plainfin midshipman experience positively-correlated increases in endocrine circulation of 17β-estradiol and testosterone during their reproductive period while 11-ketotestosterone plasma concentrations remain relatively low throughout the reproductive cycle (Sisneros et al., 2004b). Interestingly, testosterone does not act as “the male” or masculinizing hormone in fishes (Le Page et al., 2010), but instead is frequently enzymatically converted to estrogens by aromatase (Diotel et al., 2010). In addition to these associative observations, estrogenic and androgenic implantation in ovariectomized non-reproductive females corroborated the reproductive state-dependent nature of saccular afferent response properties to spectral range of the male courtship call (Sisneros et al., 2004a), directly demonstrating the role of estrogen and testosterone as modulators of saccular afferent neurophysiology. Taken together, the sensory plasticity to the vocalizations of potential mates at the level of the auditory periphery in female plainfin midshipman receivers closely parallels reproductive condition-dependent fluctuations in
circulating levels of sex hormones (Rohmann & Bass, 2011) and can be replicated by hormonal treatment (Sisneros et al., 2004a). It is unclear, however, if steroid hormones exert similar neuromodulatory effects within the central auditory system of female plainfin midshipman.

Further insight into the role of steroid hormones in mediating female reproductive state-dependent plasticity of peripheral and brainstem auditory function in fishes is provided by audiometric investigations of the round goby and *A. burtoni* auditory brainstem response to conspecific male acoustic advertisement signal and composite tonal exposure. The female *A. burtoni* exhibits a similar pattern to that of the plainfin midshipman in circulating 17β-estradiol and testosterone levels throughout their reproductive cycle, such that the serum levels of 17β-estradiol and testosterone peak during periods of reproductive receptivity (Maruska & Fernald, 2010). In parallel to this endocrinological cyclicity, the female *A. burtoni* shows decreased auditory brainstem response thresholds to the spectral components of the acoustic courtship signal of the male *A. burtoni* during the reproductive period (Maruska et al., 2012). Despite the similar estrogen-associated enhancement of acoustic mating signal-evoked auditory brainstem response in female round gobies (Zeyl et al., 2013), this hormonal dependency of auditory sensitivity does not appear to be associated with reproductive condition (Zeyl et al., 2013) as estimated by the gonadosomatic index (Zeyl et al., 2014). Specifically, increased female round goby auditory brainstem response amplitude and latency in response to male advertisement call playback and decreased auditory sensitivity to 100-200 Hz tones are associated with elevated levels of circulating estrogens while no effect of reproductive condition is observed (Zeyl et al., 2013), providing associative evidence which distinguishes the contributions of estrogens in modulating the response properties of the auditory system from that of reproductive state. Zeyl et al. (2013) posited the simultaneous enhanced sensitivity to the male courtship signal and reduced audibility of tones within the range of 100-200 Hz was attributed to a frequency filtering mechanism, improving the detection of the higher components of the male signal. The induction of prolonged auditory brainstem response latency through estrogen treatment exceeding physiologically relevant levels has been demonstrated in ovariectomized rats (Coleman et al., 1994), however, the role of response latency in auditory function remains elusive
(Zeyl et al., 2013). Nonetheless, these data suggest a role for sex steroids in mediating the plasticity of auditory circuit sensitivity beyond the auditory periphery, though our current understanding of this role is limited.

As is true in the auditory periphery of fishes, observations of the hormonal-mediated plasticity of the central auditory system are almost exclusively correlative; however, Maruska and Tricas (2011) demonstrated the central auditory system is receptive to direct gonadotrophin-releasing hormone neuromodulation. Gonadotrophin-releasing hormone 1 drives the production and release of neuroendocrine regulators, including estrogens and androgens, from the gonads through the hypothalamic-pituitary-gonadal axis while the proposed neuromodulatory (Karigo et al., 2013) gonadotrophin-releasing hormone 2 and gonadotrophin-releasing hormone 3 forms are expressed within the fish midbrain tegmentum (Kanda et al., 2010) and terminal nerve ganglia (Oka, 1992; Abe & Oka, 2000; Wayne et al., 2005), respectively. The Hawaiian sergeant fish (*Abudefduf abdominalis*) is an acoustically communicating species (Maruska et al., 2007) that exhibits year-long reproductive cyclicity despite clear seasonal variation in reproduction (Helfrich, 1958; Tyler, 1992). In this species, the auditory region of the midbrain torus semicircularis exhibits a great sensitivity to acoustic courtship signals (Maruska & Tricas, 2009) and demonstrates seasonal variation in gonadotrophin-releasing hormone innervation peaking within the post-spawning period (Maruska & Tricas, 2011). Exogenous application of both proposed neuromodulator gonadotrophin-releasing hormone forms to the torus semicircularis, an area which demonstrates seasonal variation in gonadotrophin-releasing hormone innervation peaking within the post-spawning period, induced a largely inhibitory effect on the residing neurons (Maruska & Tricas, 2011). Less inhibition of the torus semicircularis during the peak reproductive season could promote improved processing of acoustic communication signals (Maruska & Tricas, 2009; Maruska & Tricas, 2011). Accordingly, it is posited that gonadotrophin-releasing hormone modulates the auditory receptivity of courtship signals within the midbrain through inhibitory and disinhibitory mechanisms which parallel seasonal changes in reproductive activity (Maruska & Tricas, 2011). Conversely, there appears to be no relationship between reproductive state and auditory sensitivity as revealed by auditory brainstem response investigations for either male or female Hawaiian sergeant
fish, an observation mirrored by saccular potentials recorded in the Lusitanian toadfish (*Halobatrachus didactylus*) (Vasconcelos et al., 2011). Like the Hawaiian sergeant fish, the Lusitanian toadfish participates in year-round acoustic communication for purposes beyond reproduction (Amorim et al., 2006), and has consequentially been posited to circumvent reproductive state-associated auditory tuning to permit sustained audition throughout the year (Vasconcelos et al., 2011) despite having well-defined breeding period (Modesto & Canário, 2003). Additionally, the torus semicircularis of female plainfin midshipman has been shown to demonstrate a similar specificity of activation in response to conspecific male advertisement calls over ambient noise (Mohr et al., 2018) as that which was demonstrated in the Hawaiian sergeant fish (Maruska & Tricas, 2009). Despite the differences in saccular sensitivity plasticity exhibited by the Lusitanian toadfish (Vasconcelos et al., 2011) and the plainfin midshipman (Sisneros, 2009; Rohmann & Bass, 2011) a similar mechanism mediating the plasticity of central auditory system sensitivity may also exist within the plainfin midshipman. Future research is needed to examine whether the higher-order auditory nuclei of the Lusitanian toadfish and plainfin midshipman possess similar mechanisms of plasticity to that of the Hawaiian sergeant fish. The work conducted within the auditory circuit of the Hawaiian sergeant fish demonstrates the neuromodulatory capacity of gonadotrophin-releasing hormone within the auditory midbrain while also highlighting the importance of central auditory system investigations and direct experimentation to improving our understanding of the auditory function plasticity and the underlying physiological mechanisms.

The condition-dependent perception of potential competitors and the resulting auditory-evoked behaviours provide compelling evidence for the neuromodulatory role of sex hormones in promoting competitive reproductive behaviours. In the plainfin midshipman, both alternative reproductive morphs exhibit improved auditory sensitivity to 85-385 Hz, which characterize the spectral content of the higher harmonics of the type I male advertisement call (Rohmann & Bass, 2011; Bhandiwad et al., 2017). This male reproductive state-dependent alteration in receiver sensitivity is concurrent with the seasonally observed positive phonotaxis in response to the playback of simulated hums exhibited by both male morphs (McKibben & Bass, 1998). Moreover, brainstem evoked response audiometry revealed auditory physiology fluctuates as a function of male
reproductive condition within male *A. burtoni*, such that subordinate males demonstrate lower auditory thresholds for 600-800 Hz tones, corresponding to the upper spectral components of male *A. burtoni* sound production, in comparison to dominant males (Maruska et al., 2012). The quantification of systemic testosterone circulation within this species revealed hearing thresholds were positively correlated with 11-ketotestosterone and testosterone levels (Maruska et al., 2012). These androgenic-associated increases in auditory sensitivity in *A. burtoni* are corroborated by an apparent intermediate profile of auditory sensitivity exhibited in type I male plainfin midshipman transitioning to reproductive readiness which also possess the highest level of circulating testosterone (Rohmann & Bass, 2011). Taken together, androgenic-associated improvements in male hearing sensitivity appear to be facilitated by increases in circulating testosterones, although these gains in hearing appear to be acquired gradually across the transition period and are loosely correlated with auditory sensitivity during this time (Rohmann & Bass, 2011). In the round goby, males do not exhibit reproductive condition-dependent auditory thresholds to tones or the male mating call; however, brainstem evoked response audiometry revealed non-reproductive males exhibit shorter pulse latencies than reproductive males in response to 100-200 Hz tones, corresponding to the best frequency of the round goby (Zeyl et al., 2013), which may reflect the deterrence function of the male advertisement vocalization-simulating 175 Hz tone in male round gobies (Moynan et al., 2016), however additional study is necessary. Collectively, these observations implicate androgens in the cross-species mediation of acoustic communication signal sensation and perception plasticity in competitive contexts.

These findings indicate that during periods of reproductive receptivity, the peripheral and central auditory systems of fishes demonstrate enhanced sensitivity in a manner that can aid in the detection and processing of reproductive and competitive acoustic signals. Reproductive-state-dependent variations in circulating androgens and estrogens have been associated with the phonotaxic responses of female receivers to male advertisement acoustic signals through physiological and morphological actions upon the neural substrates of the auditory periphery. It is important to note, however, that the effects of hormones on the auditory periphery (Sisneros et al., 2004a) and central auditory circuit (Maruska & Tricas, 2011) have each only been directly investigated in
one study. As a result, many questions remain as to if and how hormones directly regulate plasticity of auditory function in fishes and how their effects on the auditory circuit functionality vary across a diverse array of species.

1.2.2. Morphological and Gene Expression Plasticity within the Auditory Systems is Associated with Reproductive Condition

A fundamental area of reproductive condition-dependent plasticity of auditory function is the parallel alteration of the underlying neural substrates and accessory structures which facilitate this timely flexibility of audition. Several genetic and morphological mechanisms have been implicated as mediators of the observed functional plasticity discussed above. The study of morphological and gene expression plasticity involved in the regulation of auditory processing plasticity illuminates the mechanisms governing context-dependent signaling processing in fishes.

The teleost peripheral and central auditory systems exhibit an immense capacity for aromatase B activity (Pasmanik & Callard, 1985; Callard, 1990; Forlano et al., 2001; Forlano et al., 2006; Diotel et al., 2010) and its expression is regulated by steroid hormones (Forlano & Bass, 2005b; Menuet et al., 2005; Mouriec et al., 2009; Xing et al., 2016a). Steroid-dependent increases in aromatase B expression in a cell type-specific manner is mediated through the steroid-specific response elements, well-conserved sequences within the promoter region of the aromatase B gene ($cyp19a1b$) (Diotel et al., 2010). Aromatase B is almost exclusively expressed within glia present in the fish brain (Diotel et al., 2018), with the exception of its presence within the peripheral ganglion neurons on the VIII nerve of the plainfin midshipman (Forlano & Bass, 2005a). Female and type I male plainfin midshipman fish exhibit regionally plastic expression of aromatase within the saccule across their reproductive cycle with peaks in expression coinciding with periods of reproductive readiness (Forlano & Bass, 2005a). Furthermore, exogenous testosterone or estrogen treatment alone is sufficient to induce this transcriptional reproductive phenotype in non-reproductive ovariectomized females (Forlano & Bass, 2005b). In contrast, aromatase expression within the saccule of the female $A. burtoni$ decreases with increasing circulating androgen (testosterone and 11-ketotestosterone) and estrogen levels, which are characteristic of periods of reproductive receptivity (Maruska & Fernald, 2010), corresponding to greater saccular aromatase
expression within non-reproductive females (Maruska & Fernald, 2010). However, this hormonal-association of aromatase expression was not evident in the saccule of males or the brain (Maruska & Fernald, 2010). It is important to note, however, that this investigation of aromatase expression employed an aromatase primer which closely resembled the coding region for cyp19a1a (Maruska & Fernald, 2010), an isoform of the gene responsible for aromatase B expression which is primarily expressed within gonadal tissue (Guiguen et al., 2010), and investigations adopting a primer with greater specificity for the aromatase B gene may yield different results (Maruska & Fernald, 2010). Despite this apparent cross-species divergence in the timely fluctuation of aromatase expression, the regulatory effect of sex hormones on aromatase B expression appears to facilitate modulated functional specificity through the enhancement of steroidogenic capacity within auditory targets and subsequent upregulation of estrogen synthesis locally.

Timely variation of receptor expression within the auditory system as a function of reproductive-condition and circulating hormone levels further mediates the receptivity of auditory circuit substrates to steroid hormones. Expression of saccular ERα, ERβ1, ARα, and ARβ is more pronounced in non-reproductive than gravid female A. burtoni and inversely proportional to endogenous estrogen and androgen levels (Maruska & Fernald, 2010). Concentrated expression of ERβ1 and ERβ2 within the plainfin midshipman saccule has been demonstrated within the apical surface and cytoplasm of hair cells, respectively (Fergus & Bass, 2013), while ERα expression has been localized adjacent to hair cells (Forlano et al., 2005). A similar inverse relationship was demonstrated in the saccule of male dominant and subordinate A. burtoni (Maruska & Fernald, 2010). Interestingly, receptor expression appears to be largely steroid-hormone independent within the central auditory system of female A. burtoni, with the exception of the positive association between ARα and circulating testosterone (Maruska & Fernald, 2010). Nevertheless, several central nuclei within the central auditory circuit and accessory structures exhibit steroid hormone receptivity and aromatase potential including the central posterior nucleus, torus semicircularis, and periventricular nucleus of the posterior tuberculum (Forlano et al., 2001; Forlano et al., 2005; Forlano et al., 2010; Fergus & Bass, 2013; Forlano et al., 2016), lending support to the notion that the central auditory system may be a target of direct sex hormone modulation.
Morphological alterations of the substrates underlying audition are not limited to the modification of regional steroidogenic capacity and neuromodulator receptivity within these acoustically communicating species. The saccular hair cells of the plainfin midshipman feature reproductive state-associated expression of large conductance calcium-activated potassium (BK) channels (Rohmann et al., 2009), where reproductive female saccules demonstrate an upregulation of the BK channels which parallels saccular sensitivity (Rohmann et al., 2013). Pharmacological inhibition of BK channels in reproductive males yielded auditory-evoked saccular thresholds in response to tonal stimulation which were indistinguishable from non-reproductive males (Rohmann et al., 2013). This finding is consistent with the elevation of saccular auditory thresholds in larval zebrafish in response to the suppression of BK channel gene expression (Rohmann et al., 2014), suggesting BK channel abundance is both a significant and direct facilitator of auditory tuning in fishes. In addition to their involvement in electrical signaling (Fettiplace and Fuchs 1999), BK channels have been implicated in the apoptotic and cell survival pathways (Liao et al., 2010; Sokolowski et al., 2011; Sakai & Sokolowski, 2015). It is thought reproductive condition-dependent saccular sensitivity is mediated in part by decreased and increased saccular-specific hair cell death and addition, respectively, contributing to increased hair cell density and decreased stereocilia length in reproductive female plainfin midshipman fish (Coffin et al., 2012). This finding is further supported by the identification of intersexual differences of hair cell density within the round goby, where females exhibited greater hair cell density and auditory tuning than male conspecifics (Zeyl et al., 2013). Although estrogenic signaling is speculated to regulate both reproductive receptivity-associated enhanced hair cell survival (Coffin et al., 2012) and BK channel upregulation (Rohmann et al., 2013) in the plainfin midshipman, however, these inferences remain to be tested. Together, these findings suggest BK channel abundance plays a significant role in the plasticity of tuning in the auditory periphery through electrical resonance while also potentially contributing to the regulation of hair cell density within the saccular epithelium (Rohmann et., 2013).

It is also important to consider that direct neuromodulatory action on auditory system substrates may not be the only mechanism contributing to plasticity of audition during the reproductive cycles of these species but rather, altered audibility of acoustic
signals may be an indirect consequence. For example, a recent study determined that the addition of weight to the underside of the buccal cavity of female *A. burtoni*, a mouth-brooding species, was sufficient to mimic mouth-brooding dependent (and thus reproductive-associated) alterations in swim bladder shape, possibly altering the proximity of the anterior swim bladder to the inner ear (Butler et al., 2017), a factor implicated in auditory frequency sensitivity (Lechner & Ladich, 2008; Schulz-Mirbach et al., 2012; Zebedin & Ladich, 2013; Butler et al., 2017; Mohr et al., 2017). This finding is consistent with greater swim bladder horn size in plainfin midshipman females and type II males in comparison to type I males, such that females and type II males exhibit shorter distances between the swim bladder and otolithic auditory end organs than humming males (Mohr et al., 2017). This morphometric mechanism within the female and type II male plainfin midshipman is posited to facilitate, in part, enhanced detection of type I male vocalizations (Mohr et al., 2017); however reproductive state-dependent differences in plainfin midshipman swim bladder morphology has yet to be investigated. Furthermore, recent transcriptomic analysis of the channel catfish (*Ictalurus punctatus*) swim bladder revealed genes associated with audition in fishes are expressed within the swim bladder (Yang et al., 2018). It will thus be interesting to further explore whether the swim bladder plays a significant role in the modulation of auditory responses across reproductive periods of acoustically communicating fishes.

### 1.2.3. Catecholaminergic Innervation Varies as a Function of Reproductive Condition

Catecholaminergic modulation of neurophysiological substrates underlying the seasonal plasticity of auditory sensitivity has received relatively less attention than the neuromodulatory role of steroid hormones in fishes (Forlano et al., 2016); however, recent research provides insight into the catecholaminergic innervation and neural activation within the auditory substrates involved. Catecholamines, that is, dopamine and noradrenaline, are frequently studied neuromodulators in bioacoustics (reviewed in Forlano & Sisneros, 2016; Maney & Rodriguez-Saltos, 2016), as they are involved in social behaviour, motivation, attention, and sensory processing (Aston-Jones & Cohen, 2005; Ritters, 2012; Lynch, 2017; Nienborg & Jacob, 2018). The vertebrate auditory pathway receives input from catecholaminergic circuitry (O’Connell et al., 2010; Forlano et al., 2014; Forlano et al., 2015; Forlano & Sisneros, 2016; Maney & Rodriguez-Saltos,
2016; Perelmuter & Forlano, 2017), which in turn can modulate acoustic signal reception, processing, and behavioural responses (Endepols et al., 2004; Lynch & Ball, 2008; Hoke & Pitts, 2012; Petersen et al., 2013; Forlano & Sisneros, 2016; Forlano et al., 2017; Ghahramani et al., 2018).

The primary origin of catecholaminergic inputs within the central nervous system of fishes resides within the diencephalic posterior tuberculum (Rink & Wullimann, 2001; Ryu et al., 2006; Filippi et al., 2010; Kastenhuber et al., 2010; Tay et al., 2011; Forlano et al., 2014; Forlano & Sisneros, 2016; López et al., 2019). Catecholaminergic descending projections originating from within the periventricular posterior tuberculum target multiple domains within the plainfin midshipman auditory system, including the primary auditory recipients, the descending octaval nucleus and secondary octaval population, and the octavolateralis efferent nucleus which provides cholinergic innervation to the inner ear maculae (Forlano et al., 2014; Forlano & Sisneros, 2016). Extensive descending dopaminergic projections also terminate within the saccular epithelium of zebrafish (Toro et al., 2015; Haehnel-Taguchi et al., 2018) and plainfin midshipman (Forlano et al., 2014; Forlano et al., 2015; Forlano & Sisneros, 2016; Perelmuter & Forlano, 2017), where ramifying nerve fibers have been demonstrated to target individual sensory hair cells (Forlano et al., 2014; Forlano et al., 2015; Forlano & Sisneros, 2016; Perelmuter & Forlano, 2017). Interestingly, the developing (Haehnel-Taguchi et al., 2018) and mature zebrafish swim bladder receives sympathetic noradrenergic innervation, again highlighting the potential role of the swim bladder in modulating audition (Finney et al., 2006).

In addition to the established catecholaminergic circuitry within the peripheral and central auditory systems, remarkable plasticity of catecholaminergic innervation within the auditory system is evident across the reproductive cycle of female plainfin midshipman (Forlano et al., 2015). Immunohistochemical analysis of reproductive and non-reproductive female plainfin midshipman revealed enhanced innervation density of catecholaminergic nerve fibers within the central posterior nucleus of the thalamus and lateral division of the nucleus preglomerulosus of reproductive females (Forlano et al., 2015). Similarly, the octavolateralis efferent nucleus of the female hindbrain is more richly innervated by catecholaminergic efferents during the reproductive period (Forlano
et al., 2015). The descending catecholaminergic inputs to the octavolateralis efferent nucleus are posited to regulate the seasonally plastic sensitivity of the auditory periphery indirectly through the modulation of inhibitory cholinergic inputs to the saccule (Furukawa, 1981; Forlano et al., 2015; Forlano & Sisneros, 2016; Perelmuter & Forlano, 2017). Contrastingly, the saccule and descending octaval nucleus exhibit the inverse pattern of seasonal variation of catecholaminergic innervation (Forlano et al., 2015). While the direct physiological effect of dopamine on the saccule has yet to be determined, the relatively sparse innervation of the auditory saccule demonstrated in reproductive females (Forlano et al., 2015), for which hearing is characteristically improved (Sisneros & Bass, 2003; Sisneros, 2009), is an observation consistent with the determined inhibitory effect of dopamine on the primary auditory nerve fibers of goldfish (Curti & Pereda, 2010) and zebrafish lateral line hair cells (Mu et al., 2012) and supposed inhibitory role of dopamine within the auditory periphery of the plainfin midshipman (Forlano et al., 2015; Forlano & Sisneros, 2016; Perelmuter & Forlano, 2017).

Catecholaminergic neuromodulation of the sensory circuitry in females is an important contributing factor underlying signal processing in reproductive contexts (reviewed in Lynch, 2017). Studies employing “behavioural molecular brain mapping”, a technique which visualizes neuronal activation through the immuno-labelling of the immediate early gene response in cells throughout the brain in order to identify activated neuronal substrates in response to extrinsic stimulation, (Jarvis, 2004; Mello & Jarvis, 2008; Horita et al., 2010) have allowed researchers to identify the neurochemical phenotype of activated neurons (e.g. Charlier et al., 2005; Lynch et al., 2012; Petersen et al., 2013; Forlano et al., 2017; Dai et al., 2018; Ghahramani et al., 2018). These studies provide further insight into the involvement of catecholamines in facilitating seasonally plastic socio-auditory behaviours in the plainfin midshipman (Petersen et al., 2013; Forlano et al., 2017; Ghahramani et al., 2018). Investigating the role of neuromodulation in reproductive condition-dependent phonotaxis in female midshipman, Forlano et al. (2017) correlated phonotaxic response duration with the distribution of neuronal activity within the central catecholaminergic circuitry of reproductive female plainfin midshipman exposed to male advertisement call playback. Greater activation of catecholaminergic neurons within the periventricular posterior tuberculum and
ventromedial-ventrolateral thalamic nuclei was strongly associated with extensive inspection of the playback speaker in the reproductive females, suggesting these forebrain nuclei play an active role in facilitating social reproductive interactions and auditory attention at the level of the individual within the plainfin midshipman acoustic mating system (Forlano et al., 2017). Additionally, the reproductive female central posterior nucleus of the thalamus, a recipient of seasonally enhanced catecholaminergic innervation (Forlano et al., 2015), displays neural selectivity for type I male mating calls compared to the vocalizations of a heterospecific (Mohr et al., 2018). While interspecific acoustic signal recognition and discrimination, to our knowledge, has yet to be described at the behavioural level in the female plainfin midshipman, the behavioural responses of the round goby to interspecific and intraspecific acoustic signaling demonstrate the selectivity of phonotaxis in fish (Rollo & Higgs, 2008). Furthermore, electrophysiological investigation has revealed the auditory system of the closely related Lusitanian toadfish (*Halobatrachus didactylus*) is capable of differentiating conspecific and heterospecific calls (Vasconcelos et al., 2010). These findings collectively raise the interesting possibility that seasonal variation of catecholaminergic innervation within higher-order auditory recipients may coordinate motivated auditory attention (Forlano et al., 2017) and perceptual acuity for species discrimination (Mohr et al., 2018), facilitating intraspecific communication and female reproductive behaviour during the breeding season of plainfin midshipman.

In addition to their involvement in auditory attention in females (Forlano et al., 2017), dopaminergic neurons within the periventricular posterior tuberculum and ventromedial-ventrolateral thalamic nuclei of both male morphs also respond robustly to the competitor male mating vocalization playback (Petersen et al., 2013; Ghahramani et al., 2018) and preferentially process acoustic reproductive over agonistic vocalizations in type II sneaker males (Ghahramani et al., 2018). Thus, while it is evident that the periventricular posterior tuberculum provides input to the auditory system circuitry and modulates auditory processing, the reciprocal is also true: social acoustic signals provoke periventricular posterior tuberculum activity (Petersen et al., 2013; Forlano et al., 2017; Ghahramani et al., 2018) in an acoustic signal-dependent manner (Ghahramani et al., 2018). The posterior tuberculum of larval zebrafish has been demonstrated to be
receptive to sensorimotor, translational visual, and tactile stimulation, suggesting a role for the posterior tuberculum in the integration of sensory information and promotion of behavioural responses (Reinig et al., 2017). Taken together, these findings strongly support a role for catecholamines in coordinating seasonal alterations in acoustic reproductive signal detection, processing, and behaviour in the plainfin midshipman through the plastic innervation of the peripheral and central auditory systems.

While it is clear that catecholaminergic neuronal activation and innervation are involved in the detection and processing of acoustic reproductive signals within the plainfin midshipman, the mechanisms underlying these anatomical interrelationships, with the exception of the current neuroanatomical evidence, have yet to be elucidated. Dopaminergic receptors are expressed within the saccule and utricle of rainbow trout (Drescher et al., 2010) and zebrafish (Toro et al., 2015) and are localized to the base of inner ear hair cells (Toro et al., 2015), placing the receptors within the field of transmission of the dopaminergic nerve endings innervating the saccular epithelium (Forlano et al., 2014; Forlano et al., 2015; Forlano & Sisneros, 2016; Perelmuter & Forlano, 2017). Dopaminergic activation of primary auditory afferent D1 receptors underlies the inhibition (Curti & Pereda, 2010) and ensuing signal-to-noise ratio enhancement of the VIII nerve response (Mu et al., 2012), further demonstrating the receptivity of the fish peripheral auditory structures to dopaminergic modulation. While it remains unclear whether this neurophysiological effect of dopamine is specific to the VIII nerve within the peripheral auditory system of fishes, the exclusive application of either a dopamine receptor antagonist or agonist to deflected hair cells of the lateral line, mechanosensory receptors which possess anatomical and physiological function analogous to that of inner ear hair cells (e.g. Lin et al., 2018; Uribe et al., 2018), reversibly activated and inhibited hair cell mechanotransduction, respectively (Toro et al., 2015). If the effects of dopaminergic input on mechanotransduction within the lateral line system are conserved within the inner ear, the findings presented above suggest that dopamine may mediate increased auditory sensitivity and enhance acoustic signal detection at the level of the peripheral auditory system. However, the direct effects of dopamine on the auditory sensitivity of fishes and whether auditory sensitivity varies as a function of reproductive condition remain to be elucidated.
A significant proportion of our understanding of functional plasticity in receiver acoustic processing concerns the anatomical and physiological mechanisms which facilitate it. Collectively, these findings strongly implicate neuromodulation in the reproductive state-dependent plasticity in the processing of acoustic stimuli and associated auditory-evoked behaviour in the acoustic mating systems of fishes. However, the involvement of sex steroids and catecholamines within the auditory circuitry of fishes have been exclusively studied in isolation (e.g. Sisneros et al., 2004a; Maruska & Tricas, 2011; Petersen et al., 2013; Forlano et al., 2017), while, in reality, these neuromodulators likely operate collaboratively to coordinate seasonal variations in fish audition. Investigations concerning the mediation of estrogenic effects within the songbird auditory system have identified catecholamines as likely downstream targets of estrogen (Caras, 2013; Maney & Rodriguez-Saltos, 2016), while estrogen-independent mechanisms of catecholaminergic modulation are also described (e.g. Ikeda et al., 2015). Additionally, dopamine has been demonstrated to inhibit the release of gonadotrophin-releasing hormone within the A. burtoni brain (Bryant, 2016), providing support for the potential interaction of catecholamines with the gonadotrophin-releasing hormone system in reproductive state-dependent alterations in the auditory sensitivity of fishes. Future studies are necessary to define the interrelation of the neuromodulatory pathways facilitating reproductive state-dependent auditory plasticity and their downstream effects within auditory systems of acoustically communicating fishes. Furthermore, despite the diverse array of fishes which employ acoustic communication (Ladich, 2018), investigation of auditory plasticity has been limited to a small proportion of these acoustic communication systems. Further development of our understanding of the processes underlying reproductive state-dependent auditory plasticity may be facilitated by extending our investigations to include an expansive array of fishes diverse in both life history traits and aural capabilities.

1.3. Auditory Plasticity Driven by Altered Experience

Sensory experience plays a crucial role in shaping the sensory systems of vertebrates (Buonomano & Merzenich, 1998; Holtmaat & Svoboda, 2009; Dunlap, 2016; Murray et al., 2016). Prolonged or repetitive sensory stimulation has been shown to contribute to robust morphological, physiological, and transcriptional alterations within
sensory pathways which often result in the enhancement or diminishment of sensory and perceptual processes (Ebbesson & Braithwaite, 2012; Dunlap, 2016; Jamann et al., 2018). The extensive study of the acoustic environment impact on mammals and birds has constructed compelling, rather comprehensive models of acoustic-mediated plasticity in vertebrates (e.g. Kandler et al., 2009; Kral, 2013; Schreiner & Polley, 2014; Singer et al., 2014; Chen & Yuan, 2015; Friauf, et al., 2015; Litovsky & Gordon, 2016; Woolley, 2017; Irvine, 2018; Yazaki-Sugiyama, 2018). For example, birds and mammals demonstrate alterations in gene expression (e.g. Anomal et al., 2013; Kelly et al., 2018) and brain neurochemistry (e.g. Moraes et al., 2018; Rodríguez-Saltos, 2018) following auditory stimulation. However, this literature concerning acoustic environment-induced auditory system plasticity are largely concentrated on mammals and birds, particularly during the developmental period (Dahmen & King, 2007; Woolley, 2017; Irvine, 2018). Our understanding of the effects of the acoustic environment on fishes (Papoutsoglou et al., 2007; Papoutsoglou et al., 2008; Papoutsoglou et al., 2010; Papoutsoglou et al., 2013; Monroe et al., 2015; Papoutsoglou et al., 2015; Smith & Monroe, 2016; Mickle & Higgs, 2017; Barcellos et al., 2018; Cox et al., 2018) is limited with a disproportionate focus on the peripheral implications of noise exposure. Here, I discuss the acoustic environment as a mediator of plasticity within the highly labile nervous systems of fish, the proposed primary predecessor in the evolution of modern hearing in vertebrates (van Bergeijk, 1967; Popper & Fay, 1997; Popper & Fay, 1999).

1.3.1. Environmental Noise-Induced Hearing Loss and Peripheral Damage

Plasticity within the sensory epithelium of the ear and auditory brainstem response properties of fishes can be driven by environmental insult, with acoustic overexposure and ototoxic chemicals being implicated in hearing deficits facilitated by the diminishement of the morphological and neurophysiological integrity of the auditory circuit in fishes (Coffin & Ramcharitar, 2016; Smith & Monroe, 2016). Acoustic overexposure is characterized as acute or prolonged exposure to intense or moderate sound pressure level which inflicts harm or risk of harm onto the receiver (Liberman, 2016). In the study of noise-induced trauma, anthropogenic noise is distinguished from other sound sources as a significant environmental pollutant in aquatic environments (Kunc et al., 2016). Similarly, ototoxic chemicals have been identified as emerging
pollutants which pose risks to the mechanosensory systems (e.g. Sisto et al., 2015; Coffin & Ramcharitar, 2016; Legradi et al., 2018; Young et al., 2018). Chemical ototoxicity results in damage and degradation of hair cell structure and function and consequent hearing impairments (reviewed in Coffin & Ramcharitar, 2016). The impacts of noxious environmental factors on the fish auditory system have been extensively reviewed elsewhere (Monroe et al., 2015; Coffin & Ramcharitar, 2016; Smith, 2016; Smith & Monroe, 2016) thus, this section briefly summarizes the relevant evidence from this body of work to examine the contributions of environmental noise and chemicals to the structural and functional plasticity of the auditory system.

Extreme acoustic exposure compromises the hearing abilities of fishes, as evident by extensive psychoacoustic and neurophysiological plasticity following high-intensity sound exposure. The study of acoustically-induced hearing plasticity in fishes was founded when Popper and Clarke (1976) employed psychoacoustic methods for the assessment of temporary threshold shifts in goldfish (*Carassius auratus*) in response to intense tonal stimulation, a phenomenon previously described in mammals (e.g. Davis et al., 1953; Benitez et al., 1972; Hunter-Duvar & Elliott, 1972). Their findings provided early evidence for frequency-dependent elevations of auditory thresholds as a consequence of high-intensity pure-tone exposure. Subsequently, neurophysiological studies emerged providing corroborating evidence of synthetic sound-induced temporary threshold shifts in goldfish (Amoser & Ladich, 2003; Smith et al., 2004a; Smith et al., 2004b; Wysocki & Ladich, 2005; Smith et al., 2006; Smith et al., 2011), Atlantic cod (*Gadus morhua*) (Enger, 1981), fathead minnow (*Pimephales promelas*) (Scholik & Yan, 2001a; Scholik & Yan, 2001b), and pictus catfish (*Pimelodus pictus*) (Amoser & Ladich, 2003). These findings demonstrating the dispersed effects of intense sound on the hearing capabilities of many species of fish warranted investigations into the possibility of harm posed to fishes as a result of anthropogenic noise within the natural environment. Sources of anthropogenic noise in aquatic environments which promote temporary threshold shifts in audition include boat engine noise (Scholik & Yan, 2002a), active sonar (Popper et al., 2007; Halvorsen et al., 2012; Halvorsen et al., 2013), and seismic air gun arrays (Popper et al., 2005). While there is clear evidence of sound-induced temporary threshold shifts in fishes, the parameters of noise exposure in conjunction with the species of the
receiver determines the extent of acoustic detriment, if any, following exposure (Smith & Monroe, 2016).

An important caveat in interpreting the results of such studies is that noise affects the hearing ability of fishes differentially across species, demonstrating the important concurrence of species-specific capacities and mechanisms of audition and sound characteristics in promoting noise-induced auditory plasticity. A predominant response of the fish auditory system to acoustic insult is its inherent susceptibility to acoustic detriment within the spectral range to which it is most sensitive (Popper & Clarke, 1976; Scholik & Yan, 2001b; Scholik & Yan, 2002a; Amoser & Ladich, 2003; Smith et al., 2004b; Smith et al., 2006; Smith et al., 2011; Smith & Monroe, 2016). Moreover, species possessing Weberian ossicle attachments to gas-filled accessory structures exhibit greater hearing impairments following acoustic overexposure than those which lack these specialized hearing structures [e.g. bluegill sunfish (*Lepomis macrochirus*) (Scholik & Yan, 2002b), Nile tilapia (*Oreochromis niloticus*) (Smith et al., 2004b), broad whitefish (*Coregonus nasus*) (Popper et al., 2005), largemouth bass (*Micropterus salmoides*), and yellow perch (*Perca flavescens*) (Halvorsen et al., 2013)]. Thus, inherent sensitivity to the acoustic signals used to impose acoustic trauma poses a greater predisposition of a species to acoustically-mediated auditory plasticity.

In addition to species-specific susceptibility, the degree of traumatic sound-induced plasticity in the audibility of acoustic signals is contingent upon the intensity (Smith et al., 2004b; Smith & Monroe, 2016) and duration of exposure (Scholik & Yan, 2001a; Scholik & Yan, 2001b; Popper et al., 2003; Smith et al., 2004a; Smith & Monroe, 2016). Employing the goldfish, a classic model of specialized structure-mediated hearing in fishes (Fay & Popper, 1974; Ladich & Wysocki, 2003), and Nile tilapia (*Oreochromis niloticus*), a species without such morphology, Smith et al. (2004b) explored the relationship between increasing sound pressure level exposure and the resulting temporary threshold shifts. Fish were exposed to white noise of increasing intensities and comparisons of pre- and post-noise exposure auditory brainstem responses revealed only the goldfish exhibit linear elevations in temporary threshold shifts with increasing sound pressure level exposure. Additionally, in extrapolating this relationship to data of previous studies employing ascending sound pressure levels to induce threshold shifts in
fishes (Scholik & Yan, 2001b; Scholik & Yan, 2002b; Amoser & Ladich, 2003), Smith et al. (2004b) substantiated the dependency of the degree of threshold shifts on sound intensity for species with highly sensitive, broad-range hearing capacities [i.e. goldfish (Amoser & Ladich, 2003; Smith et al., 2004b), pictus catfish (Amoser & Ladich, 2003), and fathead minnow (Scholik & Yan, 2001b)]. Furthermore, neurophysiological studies of the fathead minnow (Scholik & Yan, 2001a; Scholik & Yan, 2001b) and goldfish (Smith et al., 2004a) demonstrated the degree of white noise-induced threshold shifts is dependent upon the duration of exposure. However, while noise exposure of increasing durations results in increasing temporary threshold shifts across short-term exposures, this duration-dependent threshold elevation cannot be generalized to extended noise exposure. Instead, it appears that a species-specific asymptotic threshold shift occurs earlier during the sound treatment, such that hearing deficits demonstrated following 2 and 24 hours of noise exposure in the fathead minnow (Scholik & Yan, 2001a; Scholik & Yan, 2001b) and goldfish (Smith et al., 2004a), respectively, are greater than or equal to that demonstrated at extended exposures. It is important to note, however, that sounds of short durations can, in fact, have traumatic impacts on the auditory system if they are presented at extreme intensities [e.g. seismic air gun arrays (Popper et al., 2005)].

Collectively, these findings indicate that alterations in auditory function are modulated by the parameters of the sound stimulus exposure, with longer and louder acoustic exposures favouring auditory plasticity to a species-defined asymptotic limit.

Hair cell loss within the inner ear has been established as contributing peripheral mechanism underlying noise- and ototoxic chemical-induced plasticity in the neurophysiological responses of the central auditory system (reviewed in Monroe et al., 2015; Smith, 2016). The well-characterized, functionally important morphological alteration underlying partial hearing loss in fishes is the degeneration of auditory hair cells within the inner ear end organs (Smith et al., 2006; Ramcharitar & Selckmann, 2010; Smith et al., 2011; Uribe et al., 2013; Smith, 2016). Decreased hair cell bundle density and hair cell damage in response to acoustic exposure has been demonstrated in Atlantic cod (Enger, 1981), oscar (Astronotus ocellatus) (Hastings et al., 1996), pink snapper (Pagrus auratus) (McCauley et al., 2003), goldfish (Smith et al., 2006; Smith et al., 2011) and zebrafish (Danio rerio) (Schuck & Smith, 2009; Sun et al., 2011).
Similarly, aminoglycoside antibiotics and anti-cancer platinum-based drugs have established ototoxicity within the inner ear of fishes (Hawkins, 1976; Yan et al., 1991; Lombarte et al., 1993; Faucher et al., 2008; Faucher et al., 2009; Ramcharitar & Selckmann, 2010; Giari et al., 2012; Uribe et al., 2013); however, lateral line studies have described other ototoxic chemicals which are likely to have similar effects on auditory hair cells (reviewed in Coffin & Ramcharitar, 2016). Interestingly, both acoustic (Smith et al., 2006; Schuck & Smith, 2009; Smith et al., 2011; Sun et al., 2011; Casper et al., 2013) and chemical (Ramcharitar & Selckmann, 2010; Uribe et al., 2013) hair cell ablation studies within the fish inner ear have demonstrated spatially-distributed damage which corresponds to regionally-dependent frequency hearing loss, a feature shared with the tonotopically organized mammalian cochlea (Park et al., 2013). Future neurophysiological and genomic brain mapping investigations (e.g. Ehret & Fischer, 1991; Friauf, 1992) of spatial frequency encoding in fishes are required to determine the peripheral spatial organization of the inner ear and evaluate whether this tonotopic organization persists within the central auditory system.

Few studies (Smith et al., 2006; Ramcharitar & Selckmann, 2010; Smith et al., 2011; Uribe et al., 2013; Low & Higgs, 2015; Smith, 2016) have simultaneously assessed auditory hair cell loss and hearing loss as a consequence of acoustic and chemical insult in fishes, limiting our ability to draw conclusions concerning the direct relationship between induced hair cell loss and its effect on audition (Smith, 2016). However, these integrative studies suggest morphological changes within the inner ear end organs are typically paralleled by diminished auditory sensitivity in fishes (Smith et al., 2006; Ramcharitar & Selckmann, 2010; Smith et al., 2011; Uribe et al., 2013; Smith, 2016). Conversely, Song et al. (2008) found no damage to the ears of northern pike (Esox Lucius) and lake chub (Couesius plumbeus) although these same fishes had demonstrated temporary threshold elevations in a previous study (Popper et al., 2005). This disconnect between hearing loss and hair cell death was also highlighted in a recent anatomo-functional impact analysis of the heavy metal, cadmium, on the auditory system of the fathead minnow which showed a decrease in auditory sensitivity following sublethal cadmium exposure which was not associated with hair cell damage (Low & Higgs, 2015). As a result of this apparent contradiction and limited research, there has been a
call for a greater degree of integration in techniques and factors assessed within individual studies investigating the effects of noise on diverse fish species to enable the analysis of direct correlations between hearing and hair cell loss (Smith, 2016) with other factors associated with noise exposure (Mickle & Higgs, 2017).

Additional evidence of mechanical- and chemical-mediated plasticity within the auditory system of fishes is derived from investigations documenting the aftermath of peripheral auditory trauma. Time-point analysis of functional recovery following saccular damage has revealed the restoration of auditory thresholds can occur very rapidly, approximately 24 hours to 17 days post-assault offset (Popper & Clarke, 1976; Scholik & Yan, 2001a; Amoser & Ladich, 2003; Smith et al., 2004a; Smith et al., 2006; Faucher et al., 2009), depending on assault characteristics as discussed above. Similarly, recovery of hair cell density within the saccule typically occurs within 1 to 2 weeks (Lombarte et al., 1993; Smith et al., 2006; Faucher et al., 2009; Monroe et al., 2015). However, saccular hair cell anatomical recovery was not evident within the pink snapper, with deteriorating saccular integrity exhibited 58 days post-acoustic assault (McCauley et al., 2003), warranting the study of extended post-assault observation of saccular integrity. Nevertheless, hair cell regeneration within the saccule loosely parallels the recovery of audition (Smith, 2016) and appears to be facilitated by the upregulation of growth hormone expression following acoustic trauma (Schuck et al., 2011). Systemic administration of growth hormone appears to activate cellular mechanisms which promote cellular proliferation within the saccule, utricle, and lagena, thus supporting hair cell regeneration (Sun et al., 2011). Interestingly, functional recovery of the saccule can precede hair cell restoration (Smith et al., 2006). While it is supposed that this apparent conflict may attest to the non-necessity of complete regeneration (Smith and Monroe, 2016), another possibility exists. Post-damage recruitment of functionally receptive but previously pre-synaptically silent hair cells has been demonstrated within the zebrafish lateral line, highlighting a tremendous capacity for plasticity and potential for functional redundancy within the mechanosensory systems of zebrafish (Zhang et al., 2018). Although yet to be demonstrated within the inner ear (Zhang et al., 2018), this synaptic plasticity demonstrated in lateral line hair cells offers interesting prospects for our understanding of functional recovery of hair cells within the fish inner ear.
1.3.2. Auditory Stimulation as a Means of Environmental Enrichment

Apart from its detrimental role in hearing loss and mediation of peripheral anatomical damage (Smith & Monroe, 2016), auditory experience appears to be a key regulator in the plasticity of the central nervous system of fishes when it is enhanced through environmental enrichment (Papoutsoglou et al., 2007; Papoutsoglou et al., 2010; Papoutsoglou et al., 2013; Papoutsoglou et al., 2015; Barcellos et al., 2018).

Environmental enrichment, the classical paradigm used to investigate environment-induced plasticity, employs manipulations of animal housing conditions to promote sensory, cognitive, or motor stimulation (van Praag et al., 2000). In fishes, non-traumatic acoustic enrichment has been primarily investigated for its potential therapeutic applications in promoting animal welfare. As a complimentary measure to the conventional behavioural, morphological, and peripheral physiological indicators of animal welfare (Huntingford & Kadri, 2014), investigations concerning the implications of acoustic enhancement on the central nervous system examine alterations in the expression of neurochemical (Papoutsoglou et al., 2007; Papoutsoglou et al., 2010; Papoutsoglou et al., 2013; Papoutsoglou et al., 2015) and genetic factors (Barcellos et al., 2018) within the brain. Thus, this portion of the review will focus on two different, but complimentary, lines of research, each supporting the role of music as an auditory stimulus to promote central plasticity in fishes.

1.3.2.1. Musical Environmental Enrichment Modulates Monoaminergic Activity

A compelling series of experiments demonstrates the utility of musical environmental enrichment in modulating brain neurochemistry in fish (Papoutsoglou et al., 2007; Papoutsoglou et al., 2010; Papoutsoglou et al., 2013; Papoutsoglou et al., 2015) (summarized in Table 1.1). By exploring the plasticity of the monoaminergic systems of fishes in response to various musical enrichment regimes, researchers have demonstrated that the characteristics of the musical stimulus, the species and associated hearing capabilities of the receiver, and the duration of musical stimulation contribute to the differential activation of the monoaminergic systems in response to acoustic enrichment.

The activity of the monoaminergic systems is reflective of animal welfare (i.e. stress) and is highly responsive to the external environment. In general, fish experience serotonergic, dopaminergic, and/or adrenergic system activation in stressful environments.
and environmental enrichment exposure appears to mitigate these physiological responses to stress, suggesting an anxiolytic effect of environment enrichment (Øverli et al., 2001; Höglund et al., 2005; Øverli et al., 2005; Batzina et al., 2014a; Batzina et al., 2014b; Vindas et al., 2018). Furthermore, environmental enrichment has been demonstrated to reduce brain serotonergic activity independent of exposure to stressful stimuli (Höglund et al., 2005). Collectively, this research identifies musical environmental enrichment as a driver of central monoaminergic plasticity in fishes and suggests an important role of musical enrichment regime and receiver characteristics in mediating this plasticity.

Environmental enrichment in the form of musical transmission promotes whole brain monoaminergic neurochemical plasticity in fishes, particularly in a musical composition- and species-dependent manner. Three studies investigated this musical composition-mediated differential regulation of monoaminergic system activity in the gilthead seabream (*Sparus aurata*) (Papoutsoglou et al., 2015), rainbow trout (*Oncorhynchus mykiss*) (Papoutsoglou et al., 2013), and common carp (*Cyprinus carpio*) (Papoutsoglou et al., 2010) by exposing each studied species to long-term acoustic enrichment regimes featuring 4 hours of sound exposure daily. Notably, the dopaminergic systems of the gilthead seabream and rainbow trout demonstrate a similar response to musical stimulation (Papoutsoglou et al., 2013; Papoutsoglou et al., 2015). Exposure of the gilthead seabream to Wolfgang Amadeus Mozart’s musical composition, “Eine Kleine Nachtmusik”, significantly increased whole brain dopamine levels while simultaneously showing a decrease in dopaminergic turnover in comparison to silent controls (Papoutsoglou et al., 2015). In accordance with the gilthead seabream, the rainbow trout demonstrated a noteworthy, although non-significant, increase in dopamine accumulation and a significant decrease in dopaminergic metabolism (Papoutsoglou et al., 2013). Furthermore, acoustic enrichment regimes administering Nicolas de Angelis’ musical piece, “Jeux Interdits”, imposed a decrease in dopaminergic turnover in the gilthead seabream and rainbow trout, respectively, however this finding was non-significant in the latter species (Papoutsoglou et al., 2013; Papoutsoglou et al., 2015). It is important to consider, however, that these musical transmissions were ineffective in modifying the dopaminergic neurochemistry of the common carp using this particular
schedule of delivery (Papoutsoglou et al., 2010), suggesting the effects of auditory enrichment may be species-specific. Additionally, white noise (200-3700 Hz) was insufficient to induce plasticity in dopaminergic activity in the rainbow trout and gilthead seabream (Papoutsoglou et al., 2013; Papoutsoglou et al., 2015). A similar ineffectiveness of Johann Sebastian Bach’s Violin Concerto No. 1. in promoting alterations in dopaminergic activity was seen in the gilthead seabream (Papoutsoglou et al., 2015). Nevertheless, these observations collectively raise the interesting possibilities that musical pieces may have differential implications for the central dopaminergic system of fishes dependent on the inherent acoustic characteristics of the music and that the effects of musical transmissions may be species-specific.

In contrast to the congruent effects of acoustic enrichment on the dopaminergic systems of the gilthead seabream and rainbow trout (Papoutsoglou et al., 2013; Papoutsoglou et al., 2015), the serotonergic system demonstrates seemingly discordant activity in response to musical transmission across these two species. For instance, experiencing Mozart’s composition significantly increased serotonin levels in rainbow trout while the gilthead seabream reacted to Bach’s composition and white noise, such that the tissue concentrations of serotonin showed a non-significant decrease in response to each of these musical transmissions (Papoutsoglou et al., 2013; Papoutsoglou et al., 2015). However, when the different acoustic stimuli employed are considered as distinct, rather than within the overarching category of music, the influence on the serotonergic activity imposed by these acoustic exposures suggests similarities in the effects of Bach’s piece and the white noise stimulus, and the musical compositions of de Angelis and Mozart. Exposure to Bach’s “Violin Concerto No. 1” and white noise on brain neurochemistry. For example, exposure to Bach’s “Violin Concerto No. 1” and white noise suppressed the serotonergic metabolism within the brain of the gilthead seabream, evident through the significant decrease in the serotonin metabolite, 5-hydroxyindoleacetic acid (Papoutsoglou et al., 2015). Conversely, serotonin turnover, a value determined by the ratio of 5-hydroxyindoleacetic acid and serotonin concentrations within the brain, was seemingly upregulated in gilthead seabream exposed to white noise (Papoutsoglou et al., 2015). This apparent contradiction is likely a consequence of the distinctive and rather substantial, non-significant decrease in serotonin accumulation
established by long-term white noise exposure in this species (Papoutsoglou et al., 2015), contributing to an apparent consequential ratio of serotonin turnover (Øverli et al., 2005). Rainbow trout demonstrated the inverse reaction to the compositions of Mozart and de Angelis, such that both 5-hydroxyindoleacetic acid accumulation and serotonin turnover increased in comparison to silent controls to each musical composition, respectively (Papoutsoglou et al., 2013). It is important to note that de Angelis’ composition was implicated in significantly higher serotonergic turnover than that imposed by exposure to Mozart’s piece in this species (Papoutsoglou et al., 2013), however, this finding is likely attributable to the sole, substantial increase of serotonin accumulation (Øverli et al., 2005). Thus, although susceptibility to acoustic enrichment mediated-serotonergic plasticity appears to be species-dependent, environmental enrichment regimes featuring Bach’s composition and white noise appear to suppress serotonergic activity while auditory enrichment facilitated by the musical compositions of Mozart and de Angelis impose the opposite effect.

Interestingly, the common carp, possessing the broadest and most sensitive hearing capabilities of the fishes investigated (Popper, 1972; Kojima et al., 2005; Maiditsch & Ladich, 2014), showed the least monoaminergic modulation in response to musical stimulation (Papoutsoglou et al., 2007; Papoutsoglou et al., 2010). Cumulatively, the conflicting findings of two studies (Papoutsoglou et al., 2007; Papoutsoglou et al., 2010) investigating the effects of music on the whole brain neurochemistry of the common carp imply the effects of music on dopamine catabolism may be duration-dependent. Common carp that were exposed to a 122 dB (re 1 μPa) recording of Mozart’s “Eine Kleine Nachtmusik” for three 30-minute exposures daily for 84 days demonstrated a decrease in whole brain levels of the dopamine metabolite, homovanillic acid, below that of silent controls while a similar auditory enrichment regime with 60-minute musical exposures induced an increase in homovanillic acid accumulation within the brain (Papoutsoglou et al., 2007). In contrast, common carp subjected to 4 hours of daily musical enrichment delivered through either a recording of Mozart’s musical piece or Nicolas de Angelis’ “Jeux Interdits” at a sound pressure level of 130 dB (re 1 μPa) for 106 days showed no differential monoaminergic responses when compared to silent controls (Papoutsoglou et al., 2010). The apparent discrepancy of these results may be
attributed to the disparity of the sound pressure levels employed and/or the durations of musical exposure periods. In the first study, Papoutsoglou et al. (2007) subjected fish to a total of 1.5 or 3 hours of musical stimulation daily while Papoutsoglou et al. (2010) employed 4-hour music exposure periods. Interestingly, exposure to Mozart’s composition for a cumulative duration of 1.5 hours daily suppressed the accumulation of homovanillic acid, while its concentration within the brain was upregulated and unaffected by 3 and 4 hours of the same daily acoustic enrichment, respectively (Papoutsoglou et al., 2007; Papoutsoglou et al., 2010). Thus, these findings suggest a possible music exposure duration dependence of dopamine metabolism favouring intermediate duration musical exposure periods. However, the common carp studied in Papoutsoglou et al. (2010) were administered acoustic enrichment at a greater intensity and longer duration than those featured in Papoutsoglou et al. (2007), and thus experienced a greater threat of temporary threshold shift (Smith & Monroe, 2016). The common carp displays highly sensitive, broad frequency range [i.e. 50-4000 Hz (Popper, 1972; Kojima et al., 2005; Maiditsch & Ladich, 2014)] hearing capabilities which increase its susceptibility to acoustically-induced hearing deterioration (Smith & Monroe, 2016), thus impairing the audibility of the music and inhibiting the potential effects of musical environmental enrichment on brain neurochemistry. Therefore, while evidence derived from these companion studies may suggest the effects of musical enrichment on dopaminergic activity within the brain are duration-dependent, further research on this topic is warranted. Nonetheless, the differential modulation of neurochemicals in response to auditory enrichment within the common carp reinforces the importance of species-specific considerations when selecting acoustic stimuli to promote plasticity.

1.3.2.2. Musical Environmental Enrichment Promotes Transcriptional Responses

The studies examined above indicate acoustic environmental enrichment plays an important role in regulating the central neurochemistry of fishes. A separate line of research, however, concentrating on the transcriptional flexibility of the fish central nervous system in response to melodic environmental enrichment, reveals the enhancement of the auditory environment through the addition of music also contributes to gene expression-dependent neuroplastic processes within the brains of fish. Barcellos et al. (2018) exposed zebrafish (Danio rerio) to various compositions written by Antonio
Vivaldi for two 2-hour periods daily for 15 days. Real-time quantitative polymerase chain reaction analysis (RT-qPCR) revealed music exposure as a means by which environmental enrichment suppressed the whole-brain expression of interleukin-1β (il-1β) and interferon-gamma (ifn-γ), while enhancing the expression of brain-derived neurotrophic factor (bdnf) expression levels. The upregulation of pro-inflammatory cytokines, il-1β and ifn-γ, has been implicated in promoting immune-mediated plasticity within the fish brain (Huising et al., 2004; Kyritsis et al., 2012; Filiano et al., 2016; Bosak et al., 2018) and, more recently, researchers have described an intimate interplay between the immune and nervous systems in regulating social and exploratory behaviour in zebrafish (Kirsten et al., 2018). Thus, the musical exposure-dependent suppression of the pro-inflammatory response demonstrated within the zebrafish brain (Barcellos et al., 2018) may contribute to central neuroplastic processes and behavioural modification of fishes. Furthermore, the demonstrated bdnf transcriptional flexibility in response to exposure to chronic musical transmission within the zebrafish brain corroborates previous reports of the upregulation of bdnf expression facilitated by musical experience in rodents (Angelucci et al., 2007; Li et al., 2010; Marzban et al., 2011; Xing et al., 2016). Taken together, these findings suggest that the enhancement of auditory environment promotes transcriptional flexibility within the fish brain which may act to diminish stress responsivity of the central nervous system.

Several lines of evidence support the role of the external environment in the regulation of transcriptional flexibility within the central nervous system of fishes. Brain-derived neurotrophic factor is expressed within the mature and developing brain of fishes (De Felice et al., 2014; Manuel et al., 2015; Cacialli et al., 2017; Barcellos et al., 2018; Hall & Tropepe, 2018a) and is involved in the mediation of neuroprotection, synaptic plasticity, neurogenesis, and long-term potentiation (Mattson et al., 2004; Fritsch et al., 2010; Gray et al., 2013; Hall & Tropepe, 2018a). Additionally, bdnf expression is highly responsive to environmental stressors, and thus has been implicated in the central nervous system stress response in fishes (Tognoli et al., 2010; Pavlidis et al., 2015; Mes et al., 2018; Vindas et al., 2018). Acute and chronic exposure to stressors promote differential expression of bdnf, such that fish exposed to acute and chronic stress typically demonstrate an upregulation and downregulation of bdnf mRNA expression, respectively.
Interpreting the enhanced expression of \textit{bdnf} within the zebrafish brain following non-traumatic chronic music exposure with respect to the stress-exposure paradigm-dependent regulation of fish whole brain \textit{bdnf} expression, suggests musical stimulation, or at least acoustic stimulation, does not appear to inflict the stereotyped transcriptional chronic stress response. Alternatively, enhanced auditory (Barcellos et al., 2018) and visual (Hall & Tropepe, 2018a) experience appear to similarly effect \textit{bdnf} expression in zebrafish. When exposed to full spectrum light on a 14:10 light/dark cycle, larval zebrafish exhibit increased BDNF production within the optic tectum, while visual deprivation results in the opposite response (Hall & Tropepe, 2018a). This elevation of BDNF expression in response to enhanced visual experience is posited to be a consequence of experience-induced neural activity which contributes to neuronal survival within the visual processing niches within the fish brain (Hall & Tropepe, 2018a). While additional research on investigating the effects of various forms of environmental enrichment on diverse species of fish may provide us with a better understanding of the influence of environmental enrichment on \textit{bdnf} expression within the central nervous system, the differential expression of genes associated with brain cell proliferation to diverse forms of environmental enrichment has received relatively more attention (Dunlap, 2016). For example, Salvanes et al. (2013) discovered increased brain \textit{neurod1} mRNA expression in captive Atlantic salmon (\textit{Salmo salar}) following environmental enrichment exposure facilitated by the addition of fronds to the tank environment. Similarly, proliferating cell nuclear antigen (PCNA), a common proxy for cellular proliferation within the fish brain (e.g. von Krogh et al., 2010; Manuel et al., 2015; Dunlap, 2016; Dambroise et al., 2017; Lai et al., 2017), demonstrates enhanced expression within the fish brain following exposure to environmental enrichment, such as increased structural complexity of the environment (von Krogh et al., 2010; Manuel et al., 2015), and physical activity (Hall & Tropepe, 2018b). Despite the extensive examination of environmental acoustic noise on the peripheral nervous system of fishes, the effects of sound on the transcriptional profile of the central nervous system have been superficially studied (Barcellos et al., 2018). While it is clear that musical auditory experience can alter \textit{bdnf} transcription within the central nervous system of fishes, future
studies are needed to determine the precise functional role of BDNF within the brain of music-exposed fish, the acoustic characteristics of music responsible for the altered transcription of \textit{bdnf}, and whether the auditory environment promotes differential expression of additional plasticity-associated genes.

\textbf{1.4. Concluding Thoughts}

The study of auditory plasticity in fishes has yielded a wealth of knowledge concerning the underlying mechanisms and auditory substrates responsible for its facilitation. There is evidence that hormonal and catecholaminergic neuromodulation (Forlano et al., 2016) and related morphological alterations (Rohmann et al., 2009; Coffin et al., 2012; Rohmann et al., 2013; Butler et al., 2017; Mohr et al., 2017) of the auditory system and accessory substrates can promote the detection of acoustic communication signals in reproductive and competitive contexts in fishes, presumably harmonizing auditory system function with the salient characteristics of the acoustic environment of fishes (Zeyl et al., 2013; Forlano et al., 2016; Ghahramani et al., 2018). The auditory system also demonstrates plasticity in response to the environment, altering its functionality in response to traumatic noise and ototoxic chemical assault (Coffin & Ramcharitar, 2016; Smith & Monroe, 2016). Additionally, the auditory environment appears to elicit transcriptional (Barcellos et al., 2018) and neurochemical (Papoutsoglou et al., 2007; Papoutsoglou et al., 2010; Papoutsoglou et al., 2013; Papoutsoglou et al., 2015) responses within the brain of fishes through musical stimulation. Through the integration of these specialized fields within the study of audition in fishes, we have gained significant insight into the genetic and molecular mechanisms which facilitate functional auditory plasticity.

However, our present understanding of auditory system plasticity in fishes is one-dimensional, focusing primarily on the mechanistic perspective of plasticity in the auditory function of few species. For example, individual differences in responsiveness of reproductive female plainfin midshipman to the male conspecific call playback appears to be attributed, at least in part, to catecholaminergic neuromodulation; however, we have yet to directly investigate the adaptive function of this plasticity in audition (Forlano et al., 2017). Additionally, our understanding of mechanisms underlying auditory plasticity is derived from investigations of the peripheral auditory structures
(e.g. Sisneros & Bass, 2003; Sisneros et al., 2004a; Rohmann et al., 2013; Coffin & Ramcharitar, 2016; Smith & Monroe, 2016), as opposed to the central mechanisms governing auditory plasticity (e.g. Maruska & Tricas, 2011; Barcellos et al., 2018). Future studies should explore both how acoustic experience and reproductive state-associated neuromodulation modifies and modulates the brain through the direct manipulation of physiological and genetic mechanisms in acoustic receivers. Furthermore, in exploring the intricacies of the auditory system plasticity of fishes and its ecological significance, we must also investigate the adaptive significance and phylogenetic patterns of auditory plasticity. Through the examination of species diverse in aural capabilities and life history traits, we can better understand the evolution of auditory plasticity and its functional significance. For example, the study of the Lusitanian toadfish revealed not all species of acoustically communicating fishes exhibit reproductive state-dependent plasticity of hearing (Vasconcelos et al., 2011) and prompted questions concerning the evolutionary mechanisms driving the diversification of auditory plasticity (Caras, 2013). By extending our investigations of auditory system plasticity in fishes to the central auditory system in a diverse array of species, we can bridge the gap between peripheral and higher-level auditory system plasticity and gain a more comprehensive understanding of the functional significance of auditory plasticity.
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Nachtmusik” and “Romanza”) combined with light intensity, using recirculating water system. *Fish Physiology and Biochemistry*, 36(3), 539-554.


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### Tables

| Species | Common Carp  
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<td></td>
<td>(Cyprinus carpio)</td>
<td>(Sparus aurata)</td>
<td>(Oncorhynchus mykiss)</td>
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<td>100-500 Hz (Halvorsen et al., 2012; Popper et al., 2007; Wysocki et al., 2007)</td>
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#### Environmental Manipulation

<table>
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<tr>
<th>Stimuli</th>
<th>Mozart’s “Eline Kleine Nachtmusik” (10-3700 Hz)</th>
<th>Nicolas de Angelis’ “Jeux Interdits”</th>
<th>Nicolas de Angelis’ “Jeux Interdits”</th>
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<tr>
<td>Stimulus Intensity (re 1μPa)</td>
<td>122 dB</td>
<td>130 dB</td>
<td>140 dB</td>
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<tr>
<td>Exposure Duration</td>
<td>3 x 30- or 60-min exposures 5 days/week for 84 days</td>
<td>4 hrs/day, 5 days/week for 106 days</td>
<td>2- or 4- hour exposures 5 days/week for 98 days</td>
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<tr>
<td>Visual</td>
<td>80 lux</td>
<td>200 lux</td>
<td>12:12 light: dark cycle</td>
</tr>
<tr>
<td>Exposure Duration</td>
<td>0:24 or 12:12 light: dark cycle for 84 days</td>
<td></td>
<td>12:12 light: dark cycle</td>
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<tr>
<td>Species</td>
<td>Common Carp</td>
<td>Gilthead Seabream</td>
<td>Rainbow Trout</td>
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<tr>
<td><strong>Hearing Range</strong></td>
<td>100-4000 Hz (Maiditsch &amp; Ladich, 2014; Kojima et al., 2005; Popper, 1972)</td>
<td>~50-600 Hz (Fay, 1988)</td>
<td>100-500 Hz (Halvorsen et al., 2012; Popper et al., 2007; Wysocki et al., 2007)</td>
</tr>
<tr>
<td><strong>Brain Neurochemical Response</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td><strong>Dopaminergic</strong></td>
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</tr>
<tr>
<td>DA</td>
<td>NEM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DOPAC</td>
<td>NEM</td>
<td>NEM</td>
<td></td>
</tr>
<tr>
<td>HVA</td>
<td>Fish exposed to Mozart for 3 x 30 min and 3 x 60 min showed significantly lesser and greater HVA than silent controls, respectively</td>
<td>NEM</td>
<td>NEM</td>
</tr>
<tr>
<td>DOPAC: DA</td>
<td>NEM</td>
<td></td>
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</tr>
<tr>
<td>HVA: DA</td>
<td>NEM</td>
<td></td>
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</tr>
<tr>
<td>Species</td>
<td>Common Carp</td>
<td>Gilthead Seabream</td>
<td>Rainbow Trout</td>
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</tr>
<tr>
<td><strong>Hearing Range</strong></td>
<td>100- 4000 Hz (Maiditsch &amp; Ladich, 2014; Kojima et al., 2005; Popper, 1972)</td>
<td>~50-600 Hz (Fay, 1988)</td>
<td>100-500 Hz (Halvorsen et al., 2012; Popper et al., 2007; Wysocki et al., 2007)</td>
</tr>
<tr>
<td><strong>Citation</strong></td>
<td>Papoutsoglou et al. (2007)</td>
<td>Papoutsoglou et al. (2010)</td>
<td>Papoutsoglou et al. (2015)</td>
</tr>
<tr>
<td>Brain Neurochemical Response</td>
<td>Dopaminergic</td>
<td>Serotonergic</td>
<td>Noradrenergic</td>
</tr>
<tr>
<td></td>
<td>DOPAC + HVA: DA</td>
<td>5-HT</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>NEM</td>
<td>NEM</td>
<td>NEM</td>
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<tr>
<td></td>
<td>Mozart, Romanza significantly decreased dopaminergic turnover from silent controls</td>
<td>-Sound induced a NS decrease in 5-HT from silent controls; NS notable decrease in fish exposed to white noise and Bach; Bach&gt; white noise</td>
<td>Mozart significantly increased 5-HT</td>
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<td>NEM</td>
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<tr>
<td></td>
<td>Bach and white noise induced significant decrease in 5-HIAA from silent controls</td>
<td>5-HIAA: 5-HT</td>
<td>White noise caused a significant increase in serotonergic activity</td>
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<td>NEM</td>
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</table>
Table 1.1. Summary of auditory (musical) environmental influences on the dopaminergic, serotonergic, and noradrenergic brain neurochemistry of fishes. Musical experience drives dopaminergic [dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA)] and serotonergic [serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA)] brain tissue concentrations and turnover (dopaminergic: DOPAC: DA, HVA: DA, and DOPAC + HVA; serotonergic: 5-HIAA: 5-HT) in a musical composition- and species-specific manner, while there is no effect of musical stimulation (NEM) on noradrenaline (NA) brain concentrations across the examined species. Cross-modal influences (i.e. acoustic and visual) may exert combined plastic effects on the brain neurochemistry of fishes. Grey coloured areas indicate the parameter of interest was not investigated by the respective study.
CHAPTER 2
EVIDENCE FOR SOUND-INDUCED NEUROPLASTICITY: LONG-TERM PASSIVE EXPOSURE TO MODERATE-LEVEL SOUNDS INFLUENCES GENE EXPRESSION WITHIN THE ZEBRAFISH BRAIN

2.1. Introduction

The ability of the central nervous system to adjust to alterations within the external environment is widespread in vertebrates (van Praag et al., 2000). Though the vertebrate brain is capable of functionally acclimating to environmental conditions and experiences, the mechanisms of neuroplasticity, specifically neurogenesis (Barker et al., 2011), and its extent can vary across taxa or even genetic lines within a species (Monroe et al., 2016). Unlike mammals and birds which experience limited neurogenesis within the adult brain (Paredes et al., 2016), widespread cellular proliferation within the fish brain persists throughout the lifespan (Kempermann, 2016). The central neurogenesis of fishes is associated with variations in gender (Ampatzis & Dermon, 2007; Ampatzis et al., 2012; Arslan-Ergul & Adams, 2014; Karoglu et al., 2017), age (Arslan-Ergul & Adams, 2014; Karoglu et al., 2017), body length, and weight (Leonard et al., 1978; Birse et al., 1980; Leyhausen et al., 1987; Brandstätter & Kotrschal, 1989; Brandstätter & Kotrschal, 1990; Zupanc & Horschke, 1995; Kaslin et al., 2008). In addition to these intrinsic associates of central neurogenesis, numerous studies have demonstrated the propensity of the highly pliable fish brain to modulate brain cell proliferation in response to environmental influences (reviewed in Dunlap, 2016).

Environmental enrichment has been widely used to study neuroplasticity within the central nervous system of fishes (Ebbesson & Braithwaite, 2012; Dunlap, 2016). Neuroplasticity refers to the alteration of the functional, physiological, and morphological characteristics of the nervous system in response to experience (Ebbesson & Braithwaite, 2012). Various ecological conditions [e.g. social stress (Dunlap et al., 2011; Johansen et al., 2012; Maruska et al., 2012; Sørensen et al., 2012; Lindsey & Tropepe, 2014; Øverli & Sørensen, 2016), habitat structure (von Krogh et al., 2010; Dunlap et al., 2011; Salvanes et al., 2013), and predation (Dunlap et al., 2016; Dunlap et al., 2017)], often studied to promote animal welfare, have demonstrated an ability to significantly influence the broad neurogenic outcome of the central nervous system. However, these ecological
conditions are often associated with multimodal stimuli and thus contribute to widespread effects on brain cell proliferation (Lindsey et al., 2014; Dunlap, 2016). Alternatively, sensory modality-specific manipulations of the visual spectral and chemosensory environments have been shown to independently elicit regionally-specific effects on cellular proliferation, generally distributed within the relevant sensory processing areas of the fish brain (Lindsey et al., 2014). While environmental enrichment studies of electreoreception (Dunlap et al., 2008), vision (Lindsey et al., 2014; Hall & Tropepe, 2018a), and chemoreception (Chung-Davidson et al., 2008; Lindsey et al., 2014) have demonstrated an ability to stimulate neurogenesis within the adult brain through sensory exposure, there is limited knowledge concerning the effect of acoustic stimuli on the proliferative-capability of the central auditory system of fishes.

While fish monitor their environment for ecologically relevant acoustic signals (Fay & Popper, 2000), they are also exposed to numerous anthropogenic sound sources that fall within their audible range (Hawkins & Popper; 2018). Consequently, there is significant interest in delineating the consequences of altered soundscape on fish physiology, morphology, behaviour, and overall welfare (Mickle & Higgs, 2017; Cox et al., 2018). Investigations of the potential morphological and functional implications of aquatic anthropogenic noise have determined high-intensity sound promotes increased cellular apoptosis (Smith et al., 2006) and sensory hair cell damage in the auditory periphery (Hastings et al., 1996; Smith et al., 2006; Smith et al., 2011; Schuck & Smith, 2009; Casper et al., 2013), and decreased hearing sensitivity (Scholik, & Yan, 2001; Scholik & Yan, 2002; Amoser & Ladich, 2003; Smith et al., 2004a; Smith et al., 2004b; Popper et al., 2005; Wysocki et al., 2007; Crovo et al., 2015). These findings contrast with recent findings supporting classical music as a means of environmental enrichment and anxiolytic treatment to promote fish welfare in captive environments. Exposure to classical music modulates the concentration of noradrenergic (Papoutsoglou et al., 2007), dopaminergic (Papoutsoglou et al., 2007; Papoutsoglou et al., 2008; Papoutsoglou et al., 2013; Papoutsoglou et al., 2015), and serotonergic (Papoutsoglou et al., 2007; Papoutsoglou et al., 2013; Papoutsoglou et al., 2015) neurotransmitters and metabolites within the brain in a species and musical composition-dependent manner which is posited to be contingent upon species frequency detection capabilities and composition spectral
components. Additionally, experimental evidence in adult zebrafish (*Danio rerio*) suggests music exposure in captive environments may decrease transcription of pro-inflammatory factors while upregulating the expression of brain-derived neurotrophic factor (BDNF) (Barcellos et al., 2018), a transcription factor responsible for promoting neurogenesis in the zebrafish central nervous system (Cacialli, et al., 2017; Cacialli, et al., 2018). However, specific auditory stimulus parameters have yet to be identified as sufficient for promoting auditory environment-mediated brain plasticity and/or cellular proliferation in fishes.

The present study investigates the effects of prolonged exposure to moderate sound pressure level tones of different frequencies on neuroplasticity within the central nervous system of fishes. This question was addressed by employing a targeted transcriptional analysis of genes associated with neuroplasticity and quantifying proliferating cell nuclear antigen (PCNA)-immunoreactive cells within the brain of adult zebrafish. Zebrafish are commonly used as a model of teleost adult central nervous system neurogenesis (Adolf et al., 2006; Grandel et al., 2006; Becker & Becker, 2008; Cacialli et al., 2017) and audition (Higgs et al., 2002; Higgs et al., 2003; Cervi et al., 2012; Monroe et al., 2016) due to their well characterized genome (Howe et al., 2013), delineated potential and mechanisms of neurogenesis (Grandel et al., 2006), well-defined auditory system anatomy (Higgs et al., 2003; Mueller et al., 2012; Wang et al., 2015a; Vanwalleghem et al., 2017) and sensitivity (Higgs et al., 2002; Higgs et al., 2003; Cervi et al., 2012; Wang et al., 2015a; Monroe at al., 2016). Zebrafish have the capacity to detect tones within the frequency range of 100-4000 Hz, showing the highest frequency sensitivity to 800 Hz (Higgs et al., 2002; Cervi et al., 2012). Specifically, the present study employed tones of 100 Hz and 800 Hz at a sound pressure level of 140 dB (re 1 μPa) for durations across a four-week period to determine if there was differential expression of factors involved in neuroplasticity and delineate the temporal patterns in the transcription of neuroplasticity genes within the adult zebrafish brain. Additionally, zebrafish swimming behaviour has been shown to be altered in response to sound exposure (Neo et al., 2015; Sabet at al., 2015; Sabet at al., 2016a; Sabet at al., 2016b; Sabet at al., 2016c) and influence cellular proliferation within the brain (Lema et al.,
Thus, an additional goal of the current study was to determine whether long term exposure to tone influenced overall swimming behaviour.

2.2. Methods

2.2.1. Animal Care

Zebrafish were obtained from a local pet store and housed in a barren, 50 cm x 25 cm x 30 cm, 37.9 L glass experimental aquarium, with a water column depth of 28 cm within a sound attenuating chamber (CL-14 LP, Eckel Noise Control Technologies, Morrisburg, ON, Canada). Fish were maintained under conditions of constant temperature (28-30°C) and a 12:12 light:dark cycle. Water in the tank was filtered with an external canister filter (Fluval 106 Canister Filter, Canada). The perimeter of the housing tank was covered with black plastic and a light was positioned above the tank. Zebrafish were acclimatized to the aquarium for 7 days prior to experiment commencement. All animal work was approved by University of Windsor Animal Care Committee in accordance with guidelines implemented by the Canadian Council for Animal Care.

2.2.2. Auditory Stimuli

Two pure sinusoidal tones (100 Hz and 800 Hz) were generated using Adobe Audition v1.5 (Adobe Systems Inc., San Jose, CA, USA) and played through a speaker (LSP-60, Monacor International, Bremen, Germany) positioned on an acoustic foam panel 10 cm away from the nearest tank wall. The speaker was connected to an amplifier (Alesis RA300, Alesis Studio Electronics, Inc., Santa Monica, CA, USA) placed outside of the sound attenuation chamber. Sound stimuli were stored and played from a MP3 player (HS-T29A-8GBMX, Hipstreet). The duration of each generated sound stimulus was 24 hours and played on repeat for the duration of the designated experimental period of each treatment group. The frequency and sound pressure level of auditory stimuli were measured within the tank by a digital oscilloscope (TDS2014C, Tektronix, Beaverton, OR, USA) equipped with a hydrophone (TC4032, Reson Inc., Goleta, CA, USA) prior to the addition of fish. Input signals were amplified 50 dB (output gain) with a voltage preamplifier (EC6081/VP2000, Reson Inc., Goleta, CA, USA). Little distortion of sound stimuli within the tanks was found (Figure 2.1) with apparent 60 Hz electrical interference in the 100 Hz playback signal (Figure 2.1A).
2.2.3. Sound Exposure Protocol

Zebrafish were exposed to continuous tones of 800 Hz or 100 Hz at a sound pressure level (SPL) of 140 dB (re 1 μPa RMS) within the aquatic environment for durations of 1 hour, 1 week, 2 weeks, 3 weeks, or 4 weeks. Controls were housed in the experimental aquarium with the same tank conditions for the same durations with the amplifier turned on while no sound was played. At the end of each exposure duration, fish were moved to a smaller tank where they were euthanized by 0.004M overdose of 2-phenoxyethanol (Higgs & Radford, 2012). The total length ($M=41.89\text{mm}; SD=4.91$) of zebrafish was measured immediately prior to decapitation. The zebrafish heads were preserved in cold RNAlater® or 4% paraformaldehyde (PFA) for future genetic or immunohistochemical analysis, respectively.

2.2.4. Tissue Preparation for Histological Analysis

Following incubation in 4% PFA at 4°C for 24 hours, the zebrafish heads were cryoprotected in 20% then 30% sucrose solution in 0.1 M phosphate buffered saline (PBS) for 6 hours and a minimum of 24 hours, respectively. Zebrafish heads were cryosectioned using a Leica CM 3050A cryostat into 30μm sagittal sections. Sections containing brain tissue were collected in two series on Fisherbrand™ Superfrost™ Plus Microscope Slides and allowed to dry at room temperature overnight prior to pre-processing storage at 4°C.

2.2.5. Immunohistochemistry, Image Acquisition, and Analysis

Brain sections were rehydrated with two 5-minute PBS washes and were subsequently incubated in normal horse serum for 1.5 hour (Vector Laboratories, Cat # PK-6102). The slides were incubated in mouse anti-PCNA antibody (Abcam, Cat # ab29; 1:500) diluted in antibody diluent (DAKO, Cat # S0809)/ 0.01% triton X-100 overnight at 4°C. Slides were then rinsed in PBS for two 5-minute periods. Sections were incubated in biotinylated horse anti-mouse secondary antibody (Vector Laboratories, Cat # PK-6102) for 1 hour followed by two 5-minute rinses in PBS. Endogenous peroxidase activity was quenched by an incubation in 3% hydrogen peroxide for 7 minutes. Slides were rinsed twice for 5 minutes in PBS and incubated in the avidin-biotin peroxidase complex (Vector Laboratories, Cat # PK-6102) for 1.5 hours. Slides were rinsed twice in PBS and incubated in 3,3′-diaminobenzidine (Vector Laboratories, SK-4100; DAB) for 5
minutes. Slides were incubated in distilled water for 5 minutes and subsequently subjected to a dehydration series: 1-minute rinses in 50% 70%, and 90% ethanol followed by two 2-minute rinses in 100% ethanol. The brain sections were cleared in xylene for two 3-minute incubations and coverslipped with Permount (Fisher Scientific, Cat #SP15).

Images were obtained from scans captured in brightfield illumination with the 20x objective of an AxioScan.Z1 slide scanner (Zeiss). PCNA-positive cells were quantified manually using ZEN lite 2.3 software (Zeiss) in the descending octaval nucleus (DON), secondary octaval population (SOP), and medial octavolateralis nucleus (MON). These brain structures were located using a topographical atlas of the zebrafish brain (Wullimann et al., 2012).

The dorsal medial proportion of the DON was selected for investigation due to its function as the primary auditory center within the cyprinid auditory system, receiving projections from the VIIIth nerve (Echteler, 1984; Mueller, 2012). Reciprocal projections are present between the DON and the SOP. The SOP receives auditory innervation directly from the DON and other auditory octaval nuclei. The DON and SOP innervate the central nucleus of the torus semicircularis (Echteler, 1984; Mueller, 2012), the midbrain sensory integration center of fishes. Thus, first and second order auditory nuclei were surveyed for proliferating cells. In addition, the MON was examined, the primary recipient of lateral line mechanosensory information from the VIIIth nerve (Wullimann & Grothe, 2013), to explore the possibility that exposure to a low frequency tone may result in long-term lateral line stimulation in addition to auditory stimulation.

2.2.6. Selection of Candidate Genes

Candidate genes were chosen to survey neuronal differentiation, proliferation, and synaptic plasticity within the zebrafish brain. Proneural genes, regulators of neurogenesis and progenitor-cell identity (Bertrand et al., 2002), including atonal homolog 1a (ato1a), neuronal differentiation factor 1 (neurod1) were selected due to their association with neurogenesis within the adult zebrafish brain (ato1a: Kani et al., 2010; neurod1: Kroehne et al., 2011). Proliferating cell nuclear antigen (pcna), a marker of mitotically active cells that is expressed within the adult zebrafish brain (Grandel et al., 2006), was also used to quantify proliferating cells within the brain. To quantify growth and
differentiation of neurons and synaptic plasticity, brain-derived neurotrophic factor (bdnf) (Huang & Reichardt, 2001) mRNA expression was quantified.

2.2.7. RNA Extraction and cDNA Synthesis

Zebrafish brains were dissected from the head and stored at -20°C prior to RNA extraction. Whole zebrafish brains were homogenized in 2 mL microcentrifuge tubes containing 0.75 mL of Trizol (ThermoFisher Scientific, Cat #15596026) and approximately 400 mg of 1 mm diameter beads. Samples were processed for total RNA isolation as per the manufacturer’s instructions. Immediately following RNA solubilization, the samples were treated with RNase inhibitor (ThermoFisher Scientific, Cat # N808011) as per manufacturer instruction and subsequently DNase 1-treated (New England BioLabs, Cat # M030MS). Isolated RNA was stored at -80°C. cDNA was synthesized from RNA using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, Cat # 4368814) as per manufacturer instructions and stored at -20°C until further processing.

2.2.8. Quantitative Real-Time Polymerase Chain Reaction

All primers used were obtained from previously published loci (Table 2.1). Assays for each sample were performed in duplicate using Power SYBR Green Mastermix according to the manufacturer’s instructions on the Applied Biosystems™ QuantStudio™ 12K Flex instrument (Thermofisher Scientific Inc., Mississauga). The thermocycling conditions were as follows: 50°C for 2 minutes followed by 95°C for 10 minutes; followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute; followed by 95°C for 15 seconds and 60°C for 1 minute.

2.2.9. Primer Amplification Efficiency and Quantification Cycle Determination

Amplification efficiency for each primer pair (Table 2.1) and the respective quantification cycle (Cq) values for each sample were calculated using the LinRegPCR program (v2017.1, http://LinRegPCR.nl). The Cq value is determined by the number of amplification cycles required to detect the fluorescence associated with the doubling of amplified fragment DNA above that of the background. The LinRegPCR output was saved to a Microsoft Excel file (v1807, Microsoft, Redmond, WA, USA) where technical replicate assays were then averaged.
2.2.10. Evaluation of Endogenous Control Genes

The endogenous control genes, β-actin and elongation factor-1a (ef1a), were selected for the normalization of candidate gene transcription data due to the determined stability of expression in zebrafish tissues across treatments and development (McCurley & Callard, 2008; Casadei et al., 2011; but see Xu et al., 2016). Two-way analyses of variance (ANOVA) were performed using SPSS to test for transcriptional responses (quantified by Cq values) of β-actin and ef1a to sound treatment, duration of treatment, and their interaction.

There was no transcriptional response of β-actin to sound treatment ($F_{2,45}=0.668$, $p=0.518$), duration of sound treatment ($F_{4,45}=0.247$, $p=0.910$), or their interaction ($F_{8,45}=1.163$, $p=0.342$). Similarly, duration ($F_{4,45}=0.308$, $p=0.871$) and sound treatment ($F_{2,45}=0.368$, $p=0.695$) had no statistically significant effect on ef1a expression nor was there evidence of an interaction between duration and auditory environment ($F_{8,45}=0.842$, $p=0.571$). In addition, the amplification efficiency of β-actin and ef1a primer sets were empirically determined to be comparable with that of each other, neurod1, atoh1a, and bdnf (Table 2.1). Thus, both β-actin and ef1a were selected as appropriate endogenous control genes for the normalization of candidate gene expression data in this study.

2.2.11. Normalization and Calculation of Log-Transformed Fold Change

Differential gene expression was evaluated by a comparison of the average log$_2$-transformed fold change in whole brain transcript abundance between fish exposed to each tone. For each sample, the geometric mean Cq value of two endogenous genes, β-actin and ef1a, was used for the normalization of candidate gene expression; generating ΔCq values for all candidate genes (Figure A.1, Appendix). To calculate the ΔΔCq value of each gene for each sample, the ΔCq values for each sample were determined relative to the group mean ΔCq value for their counterparts in the silent control treatment groups of matched durations (Figure A.2, Appendix). Fold change gene expression of 800 Hz and 100 Hz-treated fish and was obtained using the $2^{-\Delta\Delta Cq}$ method (Livak & Schmittgen, 2001), however, the respective determined mean amplification efficiency for each candidate gene across samples was used in place of the overestimated theoretical 2-fold amplification efficiency (Figure A.3, Appendix). The determined fold change values
were then log2-transformed (Figure 2.2). All calculations were performed in Microsoft Excel (v. 1807, Microsoft, Redmond, WA, USA).

2.2.12. Behavioural Analysis

To assess the potential interaction between swimming activity and gene expression, zebrafish behaviour was recorded during the last hour of 100 Hz and 800 Hz sound exposure with a GoPro Hero 3 (GoPro Inc., San Mateo, CA, USA) camera. Subsequent video analysis was conducted with Solomon Coder software (v. 17.03.22; Budapest, Hungary) to quantify the time spent swimming, coasting (passive movement with no evidence of tail or fin movements) (Kalueff et al., 2013), and out-of-sight (visually inaccessible on the video). Beginning with a segment at the onset of recording, behaviour in the 1-hour video was sampled every 9 minutes for seven 1-minute segments. For each 1-minute sampling period, the swimming behaviour of three randomly selected fish was recorded.

A three-way ANOVA conducted in SPSS revealed a significant effect of sampling period on zebrafish swimming behaviour ($F_{6,140}=8.205, p<0.001$). However, Tukey’s post hoc test revealed only the first sampling period was significantly different from the remainder ($ps<0.005$), suggesting experimental interference due to camera placement immediately prior to the commencement of the first behaviour sampling period. As a result, sampling period was disregarded as a fixed factor in statistical analyses of swimming behaviour and the first 1-minute sampling period of every subject was omitted from future behavioural analyses.

2.2.13. Statistical Analyses

Statistical analyses of transcription and swimming behaviour were performed using IBM SPSS Statistics for Windows (v. 24.0. IBM Corp, Armonk, NY: IBM Corp.) and results were plotted in GraphPad Prism for Windows (v. 8.0.2, GraphPad Software, San Diego, California, USA). Two-way ANOVA were employed to compare target gene expression (log2-transformed fold change) and swimming activity across treatment groups individually, with duration (1 hour, 1 weeks, 2 weeks, 3 weeks, and 4 weeks) and frequency (100 Hz vs 800 Hz) of sound exposure as independent variables. In the event of significant main effects, Tukey’s test was used to conduct post hoc comparisons of the means from the sound treatment durations. Target genes were determined to be
statistically significantly differentially transcribed in response to sound treatment if duration or frequency of exposure factors exhibited significance at the 0.05 alpha level. Similarly, the significance level used in the analysis of swimming activity in response to prolonged tone exposure was $p<0.05$.

2.3. Results

An examination of whole brain transcript abundance following prolonged exposure to continuous tones demonstrated notable trends in the expression of proliferative, proneural, and neuroplasticity-associated transcripts, with some genes being more affected by sound than others. All values are reported as the mean ± standard error of the mean (SEM).

2.3.1. Brain-Derived Neurotrophic Factor Transcription

There was a significant main effect of duration of sound treatment exposure (time spent in the experimental tank) ($F_{4,32}=7.887$, $p<0.001$; Figure 2.2A) on the relative expression of bdnf within the zebrafish brain. The Tukey post hoc test revealed the whole brain bdnf transcript abundance was significantly greater following 4 weeks of treatment exposure ($M=0.658 ± 0.237$ SEM) than after both sound treatment durations of 2 weeks ($M=-0.544 ± 0.070$ SEM, $p<0.001$) and 3 weeks ($M=-0.246 ± 0.159$ SEM, $p=0.002$). Brain-derived neurotrophic factor was statistically significantly differentially transcribed at the 2-week time point when compared to fish exposed to 1 hour ($M=0.285 ± 0.083$ SEM, $p=0.001$) and 1 week ($M=0.138 ± 0.182$ SEM, $p=0.031$). On average, both the 1-hour and 1-week sound treatment cohorts exhibited an upregulation of bdnf relative transcript abundance while expression was downregulated in fish exposed to sound for 2 weeks.

Though there was only a marginally significant main effect of frequency of sound exposure on brain bdnf amplicon fold change ($F_{1,32}=3.968$, $p=0.055$), this effect may have been masked by high variance of Cq among biological replicates. Fish subjected to the 100 Hz continuous tone treatment experienced an upregulation of whole-brain bdnf expression ($M=0.258 ± 0.123$ SEM), while the 800 Hz treatment cohort showed a downregulation ($M=-0.045 ± 0.097$ SEM). While both tones exhibited temporal fluctuations in gene expression across the duration of the 4-week sound exposure, fish exposed to 800 Hz showed discernable downregulation of bdnf transcription at both the
1-week ($M=-0.205 \pm 0.122$ SEM) and 3-week ($M=-0.411 \pm 0.215$ SEM) sampling time points which was unmatched by their counterparts exposed to 100 Hz for the same durations ($M=0.396 \pm 0.237$ SEM and $M=-0.080 \pm 0.231$ SEM, respectively). However, there was a non-significant interaction effect of duration of treatment and sound on the transcription of $bdnf$ ($F_{4,32}=0.576, p=0.682$). These apparent changes were not evident in the silent control trials, which exhibited a flat distribution of ΔCq values across the 4-week treatment period, with the exception of the apparent elevation in the average ΔCq evident at 4 weeks post stimulus onset ($M=-5.532 \pm 0.658$; Figure A.1A, Appendix).

2.3.2. Neuronal Differentiation Factor 1 Gene Transcription

There were no statistically significant main effects of duration ($F_{4,32}=1.052, p=0.396$) or frequency of sound treatment ($F_{1,32}=0.595, p=0.446$) and no significant interaction between frequency and duration ($F_{4,32}=0.628, p=0.646$) on $neurod1$ expression (Figure 2.2B). Across all trial durations, an average log$_2$-transformed fold change of 0.074 $\pm$ 0.146 SEM in $neurod1$ whole brain transcripts was demonstrated following exposure to 100 Hz. Conversely, fish exposed to 800 Hz showed an average downregulation of $neurod1$ expression ($M=-0.035 \pm 0.089$ SEM).

The expression of $neurod1$ following exposure to 100 Hz showed an apparent downregulation of expression following 2 weeks ($M=-0.161 \pm 0.019$ SEM) and 3 weeks ($M=-0.363 \pm 0.297$ SEM) of exposure, a temporal decrease in expression which is shared with $bdnf$ expression (Figure 2.2A). In contrast, 4 weeks of treatment with a 100 Hz continuous tone increased $neurod1$ transcription ($M=0.320 \pm 0.436$ SEM) while the 800 Hz treatment at the same duration did not induce a change in expression ($M=0.057 \pm 0.302$; Figure 2.2B). However, this treatment group demonstrated a large degree of variation in log$_2$-transformed fold change. A slight decrease in $neurod1$ expression is discernible following 2 weeks of exposure to 800 Hz ($M=-0.397 \pm 0.378$ SEM), however, this treatment group showed considerable variation. Though these apparent differences in $neurod1$ fold change were not shared with silent control groups, the fish exposed to the silent treatment for 1 week ($M=0.326 \pm 0.514$ SEM; Figure A.1B, Appendix) showed high variability in normalized transcription (ΔCq).
2.3.3. Atonal Basic Helix-Loop-Helix Transcription Factor 1a Transcription

Duration ($F_{4,32}=1.127$, $p=0.361$) and frequency ($F_{1,32}=0.137$, $p=0.714$) of sound treatment had no statistically significant effect on atoh1a log$_2$-transformed fold change nor was there evidence of an interaction between duration and frequency of sound exposure ($F_{4,32}=0.663$, $p=0.622$). In contrast to the downregulated transcriptional response of bdnf following exposure to both tones for 2 weeks, the transcriptional response of atoh1a peaked at the 2-week sampling time point, showing apparent upregulation of atoh1a transcription ($M=0.417\pm0.614$ SEM; Figure 2.2C). However, there is a large variation of transcription demonstrated within the 100 Hz cohort ($M=0.246\pm0.946$ SEM). Additionally, atoh1a expression was upregulated following a 2-week exposure to 800 Hz ($M=0.758\pm0.331$ SEM), an opposite transcriptional response to that demonstrated by neurod1 and bdnf following the same treatment. Of note, there was a high level of variance of log$_2$-transformed fold change within the groups exposed to 1-hour treatments of 100 Hz ($M=-0.793\pm0.458$ SEM) and 800 Hz ($M=-0.305\pm0.496$ SEM) which may have reduced power to detect main effects of frequency and duration of tonal exposure, or their interaction.

2.3.4. Proliferating Cell Nuclear Antigen Gene Expression

Transcriptional responses to duration ($F_{4,32}=0.345$, $p=0.846$) and frequency ($F_{1,32}=0.535$, $p=0.470$) of sound exposure did not show statistically significant differential pcna transcription (Figure 2.2D). Additionally, there was no evidence of a statistically significant interaction between frequency and duration of sound exposure ($F_{4,32}=0.864$, $p=0.496$). Regardless of sound frequency treatment, there were no discernible differences in pcna transcription across trial durations, demonstrated by a flat distribution (Figure 2.2D), and in all cases there was a relatively large amount of variation within treatments.

Regional quantification of PCNA-immunopositive cells within the auditory hindbrain revealed that the expression of PCNA protein may vary in response to sounds varying in frequency across a 4-week exposure period. Zebrafish brain regions surveyed included the primarily auditory recipients, the SOP and DON, and the lateral line system hindbrain recipient, the MON.

The number of PCNA-immunopositive cells within the DON showed discernable variation in response to all acoustic environmental conditions investigated (Figure 2.3A).
In response to the 100 Hz tone treatment, zebrafish exhibited an elevation of PCNA-immunopositive cells following exposure for 2 ($M=14.33 \pm 3.84$ SEM) and 4 weeks ($M=13.00 \pm 2.65$ SEM). Zebrafish treated with the 800 Hz tone for durations of 2 ($M=20.00 \pm 0.00$ SEM), 3 ($M=16.00 \pm 1.52$ SEM), and 4 ($M=18.333 \pm 4.10$ SEM) weeks showed an apparent increase in the number of PCNA-immunolabeled cells in comparison to their cohorts exposed to the 800 Hz tone for only 1 hour ($M=7.00 \pm 0.00$ SEM). The expression of PCNA within the cells of the zebrafish DON in response to the 4-week silence treatment exhibited an inverted u-shape pattern, such that a depression in the number of PCNA-positive cells decreased following 1 week ($M=19.00 \pm 0.00$ SEM) to at 2 ($M=9.00 \pm 0.00$ SEM) and 3 ($M=11.50 \pm 1.50$ SEM) weeks, respectively, followed by a restoration of PCNA-immunopositive cells at 4 weeks ($M=17.50 \pm 0.50$ SEM) following the onset of silence.

While the expression of PCNA within the SOP showed no apparent duration-dependent variation in response to exposure to the 100 Hz tone (Figure 2.3B), the 800 Hz treatment induced an apparent increase in PCNA-immunopositive cells following 3 weeks ($M=11.00 \pm 1.45$ SEM) of exposure in comparison to the number of PCNA-immunopositive cells at 1 hour ($M=4.00 \pm 2.00$ SEM) and 2 weeks ($M=6.00 \pm 0.00$ SEM). This apparent increase was followed by a decrease in the number of cells expressing PCNA at 4 weeks ($M=8.00 \pm 4.36$ SEM), however, this time point exhibited great variability. Additionally, fish exposed to prolonged silence demonstrated a gradual increase in the number of PCNA-positive cells from the 3-week ($M=7.50 \pm 4.50$) to 4-week ($M=11.50 \pm 0.50$) timepoints.

The MON, a recipient of mechanosensory information from the lateral line, exhibited notable variations in the number of PCNA-immunolabeled cells (Figure 2.3C). In response to the 800 Hz tone, zebrafish demonstrated an elevation of PCNA-immunopositive cells within the MON following 3 weeks of exposure ($M=33.20 \pm 5.12$ SEM). This proliferative response was not sustained as the cohort exposed to 800 Hz for 4 weeks ($M=25.667 \pm 4.37$ SEM) showed a decline in the number of PCNA-immunolabeled cells from that of the 3 weeks cohort. While the number of cells expressing PCNA within the MON of the zebrafish treated with the 100 Hz showed little variation across the 1-hour ($M=30.50 \pm 2.10$ SEM), 2-week ($M=31.67 \pm 6.06$ SEM), and
4-week ($M=30.00 \pm 1.00\ SEM$) groups, there was an apparent diminishment and elevation of cellular proliferation following 1 week ($M=22.00 \pm 0.00\ SEM$) and 3 weeks ($M=42.50 \pm 0.50\ SEM$) of 100 Hz tone stimulation. With the exception of a highly proliferative response of the MON exhibited following a 1-week exposure to silence ($M=46.00 \pm 0.00\ SEM$), zebrafish exposed to long-term silence showed no discernible change in the number of PCNA-immunolabeled cells from 2 ($M=36.00 \pm 0.00\ SEM$) to 4 ($M=34.50 \pm 0.50\ SEM$) weeks.

2.3.5. Swimming Behaviour in Response to Long-Term Pure Tone Exposure

Both the duration of tone exposure ($F_{4,170}=21.440; p<0.001$) and frequency of tone ($F_{1,170}=16.879, p<0.001$) had a statistically significant effect on zebrafish swimming behaviour, with a significant interaction effect between tone and duration ($F_{4,170}=22.532, p<0.001$). While exposure to the 800 Hz tone had no apparent effect on swimming behaviour (Figure 2.4), exposure to the 100 Hz tone resulted in a dramatic increase in swimming behaviour from 1-hour ($M=27.933 \pm 2.492\ SEM$) to 1-week ($M=48.167 \pm 1.402\ SEM$) and 2-week ($M=54.722 \pm 0.564\ SEM$) exposure durations followed by a rapid decline in swimming behaviour at 3 weeks ($M=30.822 \pm 2.091\ SEM$), demonstrating a similar activity level to that of both 1-hour and 4 weeks ($M=34.856 \pm 2.178\ SEM$) treatment groups.

2.4. Discussion

The consequences of prolonged acoustic experience on the auditory systems of fishes is seldom studied beyond the scope of acoustically-induced damage of peripheral auditory structures, despite the mounting evidence of musically-induced neurochemical (Papoutsoglou et al., 2007; Papoutsoglou et al., 2008; Papoutsoglou et al., 2013; Papoutsoglou et al., 2015) and transcriptional flexibility (Barcellos et al., 2018) within the fish brain. However, the present study demonstrates that prolonged single tone acoustic exposure affects the transcription of genes implicated in neuroplastic functions within the central nervous system of zebrafish. Here, I report a gene-specific transcriptional effect of pure tone exposure duration and provide a time course of this sound-induced transcriptional flexibility. Behavioural analysis further suggests that auditory stimulation alters central nervous system processing through the promotion of swimming behaviour in an exposure-duration dependent manner and thus, may also
indirectly contribute to the transcriptional response of the central nervous system of zebrafish to acoustic experience. As such, these findings warrant future investigations concerning the transcriptional consequences and multimodal influences of long-term acoustic exposure on the central nervous system of fishes.

2.4.1. Long-Term Sound Exposure Induces Duration-Dependent Transcriptional Flexibility in a Gene-Specific Manner

The present study employed a targeted approach to characterize the genomic response of specified genes involved in neuroplasticity within the zebrafish brain to continuous acoustic tonal stimulation. The transcriptional response of the brain to prolonged sound exposure was gene-specific, with only bdnf showing significant transcriptional flexibility in response to prolonged tonal sound exposure. It is important to note, however, that the acoustic responsivity of bdnf transcription within the zebrafish whole brain was paralleled by a similar, but non-significant, trend of neurod1 transcription while atoh1a transcription showed a non-significant inversely parallel response.

In fishes, environmental enrichment enhances proliferative activity within the brain (Dunlap, 2016); however, the expression of pcna was largely unaffected by sound exposure in the current experiment while bdnf transcription exhibited a sound exposure-modulated response. Specifically, bdnf was upregulated in response to 1 hour, 1 week, and 4 weeks of exposure to tonal stimulation while pcna showed no variation in expression across treatment conditions. Neural plasticity within the fish brain is associated with the expression of a plethora of genes (Schmidt et al., 2013), of which pcna, neurod1, atoh1a, and bdnf are prevalently associated with central nervous system neurogenesis, neurodifferentiation, and neuroprotection (Brandel et al., 2006; Kani et al., 2010; Kroehne et al., 2011; Cacialli et al., 2017; Cacialli et al., 2018; Hall & Tropepe, 2018a). The divergent patterns of bdnf transcription from that of pcna suggests that BDNF action in response to long-term sound exposure is not limited to the support of cell addition within the brain. This would not be surprising given the role of BDNF in mediating axonal branching, navigation, and regeneration within the fish visual system (Dawson et al., 2015) in conjunction with the demonstrated role of BDNF in mediating high fidelity tonotopic mapping and pure tone experience-dependent map expansion.
within the primary auditory cortex of the developing rat brain (Anomal et al., 2013). Although tonotopy within the fish brain has yet to be described, at least some fish appear to exhibit a crude tonotopic organization of hair cells within the saccule (Enger, 1981; Smith et al., 2011). The fluctuating transcription of \( bdnf \) described here within the zebrafish brain may reflect central tonotopic map plasticity in response to long-term exposure to single pure tones, and thus increased representation of the tone through the tuning of neuronal receptive fields within the central auditory system (de Villers-Sidani et al., 2007). Furthermore, the basic helix-loop-helix transcription factor, NeuroD1, is implicated in both the regulation of accurate central neuronal projection from the auditory periphery (Jahan et al., 2010) and the establishment of tonotopic organization within the auditory hindbrain of developing mice (Macova et al., 2018). Closely mirroring the transcription of \( bdnf \) within the zebrafish brain, \( neurod1 \) mRNA levels showed an apparent average increase at 1- and 4-weeks post-sound exposure onset, particularly in response to the 100 Hz tone. Thus, the paralleled patterns of \( neurod1 \) and \( bdnf \) transcription throughout the duration of sound exposure in the current work provides support for the possible role of prolonged acoustic experience in mediating connective plasticity within the central auditory system of fishes.

Brain-derived neurotrophic factor is largely implicated in experience-driven neuroplasticity in fishes (Wood et al., 2011; Ebbesson & Braithwaite, 2012; Johansen et al., 2012; Sørensen et al., 2013; Teles et al., 2016; Vindas et al., 2017; Hall & Tropepe, 2018a; Vindas et al., 2018). Enriched acoustic experience, in the form of chronic classical music exposure for a period of 2 weeks, has been previously demonstrated to upregulate \( bdnf \) transcription within the whole zebrafish brain (Barcellos et al., 2018). The transcriptional response of \( bdnf \) within the zebrafish brain to continuous pure tone exposure of the same duration demonstrated within the current study strikingly contrasts with the previously described musical environmental enrichment-dependent enhanced transcription of \( bdnf \). That is, the transcribed levels of central nervous system \( bdnf \) varied as a function of tonal sound treatment duration, with an inverted bell curve response demonstrated across a four-week period which nadired at 2 weeks post-stimulus onset. The disparity in the tone- and music-induced transcriptional response of \( bdnf \) within the zebrafish brain implies differential effects of complex and single-tone acoustic stimuli on
the fish central nervous system. Indeed, sensory environment complexity promotes central neural plasticity in fishes (Ebbesson & Braithwaite, 2012; Dunlap, 2016). For example, larval zebrafish reared in white light (full visual spectrum) experience an enhanced transcriptional response of *bdnf* within the optic tectum in comparison to their counterparts reared in dim lighting conditions (Hall & Tropepe, 2018a). The acoustic exposure employed by this earlier study featured classical music with a frequency bandwidth of 330 to 506 Hz and temporal variation (Barcellos et al., 2018), whereas the acoustic stimuli of the present study consisted of continuous pure tones. Therefore, it is possible that overall acoustic signal complexity contributes to the directionality of the zebrafish brain *bdnf* transcriptional response.

The transcriptional flexibility of *bdnf* is largely implicated in the acute (Pavlidis et al., 2015; Jantzen et al., 2016; Çomakli et al., 2018) and chronic (Pavlidis et al., 2015; Vindas et al., 2018) central nervous system stress response in fishes and is posited to mediate neural network adaptation in response to acute to moderate stress (Sørensen et al., 2013; Mes et al., 2018; Vindas et al., 2018). Transcriptional responses of *bdnf* to stress appear to be duration-dependent, with increased expression following acute stress (Pavlidis et al., 2015; Jantzen et al., 2016; Çomakli et al., 2018) and the downregulation of expression in the event of chronic stress (Sørensen et al., 2013; Vindas et al., 2018). In Pavlidis et al. (2015), time point analysis of *bdnf* expression within the zebrafish brain following 5 minutes of net chasing as an acute stressor revealed upregulated transcription at 15 to 60 minutes post-stressor exposure. This stress short-term response may help explain the results in the present study where acute 1-hour sound treatment promoted an upregulation in the expression of brain *bdnf*. However, Pavlidis et al. (2015) found chronic stress elevated the transcriptional response of *bdnf*, a finding which bears striking contrast to the results of the present study. This apparent discrepancy is likely attributable to the differences in predictability of the stressor conditions across these studies, that is, zebrafish in Pavlidis et al. (2015) experienced a varied multimodal 11-day stress paradigm while the present study employed a relatively predictable continuous unimodal tone exposure. However, consistent with the present study, juvenile gilthead seabream exposed to an unaltered series of two stressors daily for two weeks showed decreased *bdnf* transcript abundance regionally within the brain (Vindas et al., 2018). Thus,
predictability of stressful stimuli, like the complexity of the sensory environment, may play a role in determining the transcriptional stress response of the central nervous system (Sørensen et al., 2013).

Acoustic overexposure is an environmental stressor which promotes functional, morphological, and physiological alterations in fishes (Smith & Monroe, 2016; Mickle & Higgs, 2017; Cox et al., 2018). In this context, the variation of *bdnf* transcription across the duration of the incremental 4-week sound exposure period may be attributed to acoustic overexposure, particularly the downregulation of expression demonstrated at 2 weeks post-exposure onset. Despite the differential sensitivity of zebrafish to 100 Hz and 800 Hz frequencies (Higgs et al., 2001), sounds were presented to zebrafish at a sound pressure level of 140 dB (re 1µPa) while ensuring signal detection and standardization of intensity across acoustic treatments. However, the employment of this sound pressure level posed the risk of temporary threshold shifts in signal audibility (Scholik & Yan, 2002; Smith et al., 2004b). While the effects of noise exposure on the fish brain remain largely elusive, investigations of the murine auditory system implicate BDNF in the function of the noise-exposed brain. Following the onset of acoustically-induced hearing loss, the ventral cochlear nucleus within the murine auditory brainstem exhibits diminished levels of *bdnf* mRNA after 2 days with a prominent diminishment of transcription at 28 days post-narrow band noise exposure (Manohar et al., 2019) and decreased *bdnf* transcription within the rat primary auditory cortex 6 days post-pure tone exposure offset (Tan et al., 2007). It is important to note that while there is evidence of the downregulation of *bdnf* within isolated structures of the murine brain in response to acoustic trauma (Tan et al., 2007; Manohar et al., 2019), some structures experience noise-induced increases (i.e. inferior colliculus; Tan et al., 2007) or no alteration (i.e. descending cochlear nucleus; Manohar et al., 2019), suggesting the effects of noise-induced trauma on the transcription of *bdnf* are regionally-specific. Furthermore, auditory deprivation facilitated through bilateral cochlear excision in juvenile rats resulted in a decline in BDNF-immunopositive neurons within the auditory cortex at 2 weeks postsurgery followed an increase at 4 weeks relative to sham controls (Wang et al., 2017). This timely variation in the transcription of *bdnf* within the murine brain succeeding auditory afferent structure degeneration directly aligns with that of zebrafish following
long-term pure tone exposure described in the present study. Taken together, these studies suggest that the fluctuating timeline of \( bdnf \) transcription demonstrated within the present study may reflect the role of \( bdnf \) in modifying synaptic connectivity following peripheral ablation. This supposition is consistent with the recent demonstration of \( bdnf \)-mediated optic afferent regeneration and projection within the adult goldfish brain (Dawson et al., 2015). Chronic exogenous \( bdnf \) infusion of axotomized optic nerve fibers promotes time-sensitive regeneration into the tectum and branching; however, uniform administration of \( bdnf \) throughout the regenerative period resulted in premature ectopic branching (Dawson et al., 2015), highlighting the importance of timely variation in \( bdnf \) in supporting regenerative and remodeling processes within the sensory periphery and brain of fishes. Additionally, the inversely parallel temporal trend of \( atoh1a \) transcription in response to long-term sound exposure suggests \( atoh1a \) may also play a role in central nervous system plasticity following damage within the auditory periphery. Within the central auditory and lateral line systems of fishes, \( atoh1a \) is largely implicated in determining neuronal progenitor cell fate (Sassa et al., 2007; Kidwell et al., 2018). Thus, it is posited that \( atoh1a \) may contribute to the addition of specified neural contacts within the hindbrain auditory and lateral line nuclei to be occupied by the remodeling processes extending from the damaged auditory periphery, which may explain the coincident peak and nadir of \( atoh1a \) and \( bdnf \) transcription at 2 weeks-post acoustic stimuli onset within the present study, respectively.

### 2.4.2. Transcriptional Effects of Long-Term Sound Exposure are Frequency-Dependent

The auditory systems of fishes exhibit differential sensitivity to sound frequencies, contributing to the contrasting frequency-dependent acoustic trauma susceptibility and acoustically-evoked activation of the auditory circuit (Smith & Monroe, 2016). While it is not surprising that zebrafish in the current study showed marginally significant differential \( bdnf \) transcription in the response to prolonged exposure to the 100 Hz and 800 Hz tone, the possible experience-mediated sensory mechanisms underpinning the frequency-specific response of the zebrafish brain to long-term sound exposure are multifold. Firstly, acoustic overexposure may account for this frequency-specific response as the mechanosensory peripheral systems are susceptible to
acoustically-induced trauma with increased risk posed at spectral ranges of greatest sensitivity. Furthermore, differential central nervous system activation in response to 100 Hz and 800 Hz tones may provide a potential explanation for the differential bdnf transcriptional response. In contrast to the central auditory processing of the 800 Hz tone, the 100 Hz tone is a low-frequency multimodal stimulus which likely stimulated both mechanosensory pathways- the auditory and lateral line systems (Higgs & Radford, 2013). Lastly, contrasting patterns of swimming activity across the 4-week period in response to the 100 Hz and 800 Hz sound frequency treatments suggests bdnf transcription may be driven by frequency-specific behavioural responses. Taken together, differences in acoustically-induced trauma, signal modality, and auditory-evoked behaviour pose significant implications for the differential responsivity of zebrafish, both transcriptionally and behaviourally, to prolonged exposure to the 100 Hz and 800 Hz tones.

Changes to the auditory system neurophysiological response characteristics has been previously demonstrated to be a consequence of acoustic trauma within the auditory periphery (Smith & Monroe, 2016). The zebrafish auditory system is maximally sensitive to sounds of 800 Hz frequency, exhibiting an auditory threshold of 127 dB (re 1 μPa) (Higgs et al., 2001). Despite the presentation of the 800 Hz tone at a sound pressure level exceeding this auditory threshold by nearly 13 dB, a pattern of greater transcriptional flexibility of bdnf in response to the 100 Hz pure tone treatment relative to the highly audible 800 Hz sound frequency is apparent across the 4-week sound transmission regime. Goldfish possess similar hearing capacities to that of zebrafish (Higgs et al., 2001) and experience acoustic trauma and associated temporary threshold shifts in response to intense 100 Hz and 800 Hz tone exposures (Smith et al., 2004b; Schuck & Smith, 2009; Smith et al., 2011). In response to a 48-hour 100 Hz pure tone exposure, goldfish showed the greatest degree of temporary threshold shift and saccular damage relative to all other frequencies tested, including 800 Hz (Smith et al., 2011), a finding consistent with the differential transcriptional and behavioural responses of zebrafish to the 100 Hz and 800 Hz tones demonstrated within the current study. The 100 Hz tone is a multimodal stimulus, detected by both the auditory and lateral line systems, and thus acoustic overexposure may inflict damage to both mechanosensory systems,
demonstrating cumulative effects within the brain. Additional evidence for the contribution of differential acoustic trauma for the 100 Hz tone-specific effect on bdnf transcription is derived from the disparity in behavioural alteration in response to these tones. Zebrafish swimming activity fluctuated as a function of duration of sound treatment exclusively in response to the 100 Hz tone; however, this behavioural response to the 100 Hz was diminished following 2 weeks of sound exposure while the 800 Hz-exposed fish retained relatively stable levels of activity across the 4-week sampling period, suggesting plasticity in the behavioural responsiveness to the tone may be attributed to a temporary threshold shift in hearing.

2.4.3. Frequency-Specific Behavioural Plasticity Poses Implications for Central Nervous System Transcription

Behavioural measures provide further support for the influence of auditory experience on the central nervous system of fishes. Brain-derived neurotrophic factor transcription within the zebrafish brain in response to 100 Hz tone stimulation was inversely paralleled by an increase in swimming activity across the first two weeks of observation. Interestingly, the present study reports a surprising dissociation between pcna transcription and swimming behaviour. Swimming activity has been demonstrated to promote zebrafish forebrain cellular proliferation (Lema et al., 2005; Hall & Tropepe, 2018b) through the concomitant stimulation of the dorsal root ganglion (Hall & Tropepe, 2018), a cluster of primary sensory neurons within the zebrafish trunk responsible for relaying mechanosensory feedback to the central nervous system during locomotion (Knafo & Wyart, 2018). This apparent dissociation of swimming activity and pcna expression in both the immunohistochemical and transcriptional analyses may be attributed to limitations in the specificity and sensitivity encountered within the immunoassay (Figure 2.5) and the relatively low pcna primer set PCR efficiency (Table 2.1). Additionally, while zebrafish exposed to both the 100 Hz and 800 Hz tones showed sound exposure duration-dependent bdnf transcriptional flexibility within the brain, a trend for higher relative bdnf mRNA levels within the brain of zebrafish exposed to 100 Hz was also evident, although not statistically significant which may be due to a small sample size. However, due to the confounding increase in swimming behaviour, it is difficult to interpret the transcriptional response of bdnf as solely due to auditory
stimulation. Future research employing methodologies which limit dorsal root ganglion stimulation (Hall & Tropepe, 2018b) are required to evaluate the effect of prolonged acoustic stimulation on the physical activity level of fishes and determine the contributions of acoustically-evoked behaviour in promoting transcriptional flexibility within the fish central nervous system. Nevertheless, the transcriptional flexibility of zebrafish may be a consequence of tone-mediated swimming behaviour responses.

The frequency-specific behavioural plasticity exhibited by zebrafish may reflect acoustically-induced hair cell damage preventing the sensory processes underlying the coordination of swimming behaviour in zebrafish. The behavioural mechanisms underlying rheotaxis, swimming behaviour oriented within the direction of current flow, are mediated by lateral line mechanosensory inputs (Oteiza et al., 2017) and are negatively impacted by hair cell damage (Suli et al., 2012; Niihori et al., 2015). Recent research has demonstrated the susceptibility of the lateral line hair cells of zebrafish larvae to acoustic trauma imposed by exposure to a 40 kHz tone for 20-120 minutes, with maximal damage occurring 2-3 days post-exposure (Uribe et al., 2018). Presumably, prolonged continuous exposure to a 100 Hz tone would induce extensive mechanosensory hair cell damage within the zebrafish lateral line, as this structure is maximally sensitive to vibrational stimuli ranging from 50-100 Hz with decreasing responsiveness with increasing stimulus frequency (Brack & Ramcharitar, 2012). Thus, the contrasting behavioural responses of zebrafish to 100 Hz and 800 Hz tone exposure may be attributed to the differential sensory trauma of the lateral line system in response to the frequencies of the acoustic stimuli. However, it remains unclear whether the behavioural plasticity of zebrafish demonstrated across the time course of 100 Hz tone exposure can be credited to lateral line system acoustic trauma. Lateral line mechanosensory hair cell damage as a consequence of acoustic assault has only been recently investigated in zebrafish larvae employing relatively short (20-120 minutes) and intense exposures (~175+ dB re 1 μPa) to high-frequency tones (Uribe et al., 2018). The progression of noise-induced hair cell damage and recovery within the lateral line remains largely elusive; however, the functional and anatomical plasticity of the analogous auditory periphery as a consequence of acoustic noise exposure has received extensive attention. Acoustic overexposure at a sound pressure level of approximately
140 dB (re 1 μPa) for durations as short-lived as 2 to 24 hours has been demonstrated to induce functional threshold shifts measured by auditory brainstem response (ABR) which persist for less than 2 weeks in the fathead minnow (Scholik & Yan, 2002) and goldfish (Smith et al., 2004b). Additionally, goldfish continuously exposed to white noise at a sound pressure level of 170 dB (dB re 1 μPa) exhibit temporary threshold shifts in their ABR that are indistinguishable from their counterparts subjected to the same treatment for 24 hours (Smith et al., 2004b), suggesting functional recovery does not occur during lengthy noise exposure. In contrast, the diminishment of swimming behaviour exhibited following 2 weeks of exposure to the 100 Hz tone at 140 dB (re 1 μPa) suggests lateral line threshold shifts in response to acoustic overexposure may vary from the characteristic progression of noise-imposed functional plasticity of the peripheral auditory system and instead exhibit a more prolonged deterioration of mechanosensory function.

Alternatively, zebrafish show behavioural modification of their swimming behaviours in response to sound stimulation (Neo et al., 2015; Sabet at al., 2015; Sabet at al., 2016a; Sabet at al., 2016b; Sabet at al., 2016c; Barcellos et al., 2018) and recent research has highlighted the role of previous auditory experience in modulating the behavioural responses of fishes to sound exposure (Nedelec et al., 2016; Radford et al., 2016). Habituation is a process by which repetitive or sustained stimulus exposure produces a pronounced attenuation in the presentation of an innate response (López-Schier, 2019). The acoustic experience duration dependency of zebrafish swimming activity demonstrated exclusively in response to the 100 Hz frequency sound treatment implies low frequency tone exposure can drive behavioural responses in this species in a frequency-specific manner. This sound-mediated behavioural plasticity appears to be limited by a brief sound exposure window of 1 to 2 weeks, where zebrafish exhibited a peak increase in swimming activity followed by decreased levels of movement at 3 and 4 weeks comparable to levels measured at 1-hour post-stimulus onset. This diminished behavioural response may reflect the ability of zebrafish to tolerate tonal exposure in a sound frequency-specific manner. The juvenile threespot dascyllus (*Dascyllus trimaculatus*) demonstrates an attenuation of behavioural and physiological stress responses to boat noise following exposure durations of 1 and 2 weeks (Nedelec et al.,
2016). Likewise, juvenile European sea bass (*Dicentrarchus labrax*) show a similar duration-dependent effect of behaviour and physiology following long-term impulsive anthropogenic noise playback experience but not in response to noise associated with ship passing (Radford et al., 2016). While tolerance to acoustic exposure appears to be species- and sound-specific (Nedelec et al., 2016; Radford et al., 2016), it is possible that zebrafish in the current study exhibited behavioural habituation to the 100 Hz tone following 2 weeks of exposure, a time period of acoustic tolerance matched by that of the juvenile threespot dascyllus (Nedelec et al., 2016). Furthermore, the demonstration of sound-specific habituation in juvenile European sea bass (Radford et al., 2016) may explain the differential effects of long-term exposure to the 100 and 800 Hz tones on zebrafish swimming behaviour in the present study. The presentation of long-term habituation of the acoustically-evoked startle response in zebrafish larvae is transcriptionally-dependent (Roberts et al., 2016), which implies a role for transcriptional flexibility in facilitating the attenuated behavioural responses of fishes following prolonged auditory exposure. However, fish exposed to the 100 Hz tone for 4 weeks exhibited a return of the upregulated response of *bdnf* which exceeded that exhibited in response to the initial exposure. This transcriptional response was unmatched by a reestablishment of swimming activity at 4 weeks, making habituation to the 100 Hz tone an unlikely explanation for the observed differences in neuroplasticity gene transcription.

2.4.4. Study Limitations

An important limitation of the present study is the possible lack of uniformity across individuals in characteristics such as strain, age, rearing conditions, and gender. Zebrafish were attained from a local pet store, thus individual characteristic and life-history data were not available. These individual differences may be contributing factors underlying the observed within-treatment group variations in gene expression. Recent research has highlighted strain-specific differences in central nervous system basal gene expression of adult zebrafish, including differential transcription of *bdnf* across the Tupfel Long-Fin and AB strain lines (Gorissen et al., 2015). Additionally, the transcriptional response of *pcna* within the brain following inhibitory avoidance task training varies as a function of strain in zebrafish, with only the Tupfel Long-Fin strain exhibiting an upregulation of *pcna* expression (Gorissen et al., 2015). Although these
strains did not show differential expression of neurod1 (Gorissen et al., 2015), the zebrafish strain-dependent transcription of bdnf and pcna within the brain (Gorissen et al., 2015) may account for the variability present within the respective transcriptional analyses of the current study. Furthermore, individual differences in auditory sensitivity are attributable to both the strain/transgenic line (Monroe et al., 2016) and total length (Higgs et al., 2003) of zebrafish and could contribute to differential auditory processing of the acoustic stimuli, and thus potentially promote differential transcriptional responses. While increasing total length is associated with improvements in auditory sensitivity, total length is also loosely paralleled by age in zebrafish (Higgs et al., 2003). Zebrafish experience an age-related reduction in pcna, bdnf, and neurod1 expression, suggesting a diminished proliferative capacity of the aging zebrafish brain (Manuel et al., 2015). Age-associated alterations in gene expression within the zebrafish brain have been demonstrated to be sexually dimorphic (Arslan-Ergul & Adams, 2014). However, sexual dimorphisms in mitotic activity and its spatial distribution within the zebrafish brain have been demonstrated prior to senescence (Ampatzis & Dermon, 2007; Ampatzis et al., 2012). In addition to individual traits, variation in previous individual experiences may contribute to differences in the brain genomic response of individuals (Best et al., 2018). Although it is unlikely that previous auditory experience altered the function of the auditory periphery as evidence of permanent hearing loss has yet to be described in fishes (Smith & Monroe, 2016; but see McCauley et al., 2003) and fish were allowed to acclimate to the experimental tank for a recovery period of at least 7 days prior to sound exposure commencement, it is still possible that previous experience is implicated in the exhibited within-group gene expression variation. Specifically, environmental experience and early life stress can promote long-term transcriptional flexibility in fishes (e.g. Nyman et al., 2017; Best et al., 2018; Mes et al., 2018; Vindas et al., 2018), and thus may have contributed to the within-group variation captured by this study.

Studies employing whole brain transcriptional analysis risk the failure to detect transcriptional flexibility in small-scale brain regions (Mes et al., 2018). In an effort to improve the spatial resolution of our transcriptional analyses and mitigate omission of small-scale regionally-specific alterations in transcriptional activity in response to long-term pure tone exposure, an immunohistochemical assay for cellular proliferation within
the zebrafish hindbrain was employed. However, specificity of the immunohistochemical assay was not uniform across individuals and produced micro-scale staining artifacts which proved challenging to distinguish from positively-stained cells. In order to reduce the risk of a misleading result, individuals exhibiting these artifacts were omitted from analysis, contributing to the missing groups and small sample sizes observed within the immunohistochemistry results of the present study. Thus, while it is possible that the number of proliferating cells varied as a function of exposure to long-term tonal stimulation, I am unable to derive these conclusions from the data presented here.

Finally, the current study sampled locomotor activity randomly across the duration of the 4-week auditory environmental conditions. Locomotor activity was analyzed from 1-hour videos recorded at the end of each sampling timepoint and seven 1-minute periods. While zebrafish were randomly sampled from a multitude of fish housed within the experimental tank, it is possible that behaviour was quantified from a single fish for multiple time points, particularly nearing the end of the 4-week experiment. As a result, future investigations should employ tracking software alongside identification protocols to quantify long-term zebrafish behaviour while also ensuring individual zebrafish identification.

2.5. Study Conclusions and Implications

In summary, the findings of the present study support a role for the auditory environment in shaping the central nervous system of zebrafish through pure tone-induced transcriptional flexibility and provides mechanistic insights into the underlying drivers of experience-mediated neuroplasticity in fishes. The transcription of brain-derived neurotrophic factor was identified as highly responsive to long-term tonal stimulation in a duration- and frequency-dependent manner. This differential transcriptional response to sound frequency implicates both the lateral line and auditory systems in sound-mediated plasticity within the central nervous system of zebrafish and underscores the multimodal nature of low-frequency sounds. Furthermore, the transcriptional response of \textit{bdnf} within the zebrafish brain to tonal stimulation demonstrated within the present study strongly contrasts with that demonstrated in zebrafish exposed to musical stimulation (Barcellos et al., 2018), highlighting the important contribution of auditory environment complexity to central nervous system
transcriptional flexibility in fishes. Understanding the impacts of acoustic-overexposure on the auditory system has been a major goal in fish bioacoustics (Smith & Monroe, 2016). Although the contributions of the auditory environment to bdnf transcriptional flexibility is complex, the present study highlights acoustically-induced peripheral mechanosensory trauma as a potential driver of this phenomenon; however, this speculation remains to be tested. This work also demonstrates auditory environment-mediated behavioural plasticity in zebrafish and provides evidence that locomotor behaviour is differentially influenced by the frequency of tonal stimulation. Consequently, directional conclusions regarding the definitive driver of transcriptional flexibility within the present study are challenging to draw. Future investigations should continue to explore the effects of sound on the central nervous system of fishes and aim to differentiate the multimodal contributions of low-frequency sound to transcriptional flexibility within the brain.
References


98


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<td>AAGGAGATGAAGCGGTAACAAT</td>
<td>GTCTTGACAGAGGAGTGTC</td>
<td>104</td>
<td>1.74</td>
<td>van den Bos et al. (2017)</td>
</tr>
<tr>
<td>β-actin</td>
<td>AF057040.1</td>
<td>CGAGCAGGAGATGGGAACC</td>
<td>CAACGGAACGTCATTGC</td>
<td>102</td>
<td>1.89</td>
<td>McCurley et al. (2008)</td>
</tr>
<tr>
<td>ef1a</td>
<td>AY422992.1</td>
<td>CTGGAGGCCAGCTCAAACAT</td>
<td>TCAAGAAGATGACCGCTAGCAT TAC</td>
<td>86</td>
<td>1.87</td>
<td>van den Bos et al. (2017)</td>
</tr>
</tbody>
</table>

Table 2.1. Summary of gene primer sequences employed and empirically estimated polymerase chain reaction efficiencies of real-time quantitative reverse-transcription polymerase chain reaction assays. Description of candidate [atoh1a, brain-derived neurotrophic factor (bdnf), neural differentiation factor 1 (neurod1), and proliferating cell nuclear antigen (pcna)] and reference [β-actin and elongation factor-1a (ef1a)] gene primer sequences employed for real-time quantitative reverse-transcription polymerase chain reaction (PCR) analysis. The primer-specific PCR efficiencies were empirically estimated using the LinRegPCR program (v2017.1, http://LinRegPCR.nl).
Figures

Figure 2.1. forms of acoustic stimuli employed for zebrafish (*Danio rerio*) auditory environment treatments. Spectrograms of (A) 100 Hz and (B) 800 Hz pure tone acoustic stimuli employed in sound exposure experiments at a sound pressure level of 140 dB (re 1 μPa) as recorded by a hydrophone submerged within the experimental tank.
Figure 2.2. Log₂-transformed fold change in the zebrafish (*Danio rerio*) whole brain expression of neuroplasticity-associated genes in response to prolonged acoustic pure tone exposure. Transcription of whole-brain (A) brain-derived neurotrophic factor (*bdnf*), (B) neural differentiation factor 1 (*neurod1*), (C) atonal homolog 1a (*atoh1a*), and (D) proliferating cell nuclear antigen (*pcna*) was quantified by real-time quantitative reverse-transcription polymerase chain reaction analysis following exposure to 100 Hz (solid blue circles) or 800 Hz (open red circles) tones for prolonged durations (1 hour, 1 week, 2 weeks, 3 weeks, and 4 weeks). Log₂-transformed fold change values were calculated from fold change results determined from relative gene expression values normalized to the expression of the endogenous control genes *β*-actin and *ef1a*. These fold change results were calculated using LinRegPCR-estimated amplification efficiencies (*Table 2.1.*). Results are presented as individual log₂-transformed fold change values with means reported as horizontal lines with associated error bars depicting the standard error of the mean. Positive values indicate transcription of the gene was upregulated in comparison to the average transcription exhibited by silent controls exposed to the same treatment duration while negative values indicate a relative downregulation of gene expression. Two-way ANOVA with duration and frequency of sound exposure as independent variables was employed to determine the effect of sound exposure on whole-brain transcription. Different letters denote durations of sound exposure groups that exhibited statistically significantly differential transcription of the respective gene (*p*<0.05). Although excluded from all statistical analyses, we present an outlier denoted by a solid blue triangle.
Figure 2.3. Number of proliferating cell nuclear antigen (PCNA)-immunopositive cells within hindbrain auditory and lateral line system nuclei of zebrafish (Danio rerio) following long-term pure tone exposure. Zebrafish were subjected to a 100 Hz tone (blue), 800 Hz tone (red), or silence (black) for 1 hour, 1 week, 2 weeks, 3 weeks, and 4 weeks. Following exposure to the auditory environment, zebrafish brain tissue was collected, preserved, and sectioned. Immunohistochemical analysis for PCNA employing the 3,3’-diaminobenzidine peroxidase reaction revealed proliferating cells within the brain. Stained cells were quantified within the hindbrain auditory recipients, (A) the descending octaval nucleus (DON) and (B) the secondary octaval population (SOP) and the peripheral lateral line system recipient, (C) the medial octavolateralis nucleus (MON). Results are presented as individual values. Where applicable, the mean and standard error of the mean are depicted by bars and associated error bars, respectively.
Figure 2.4. Time-course of locomotor activity of zebrafish (*Danio rerio*) in response to long-term exposure to tonal sound stimulation. Zebrafish were exposed to pure tones of 100 Hz (blue solid circles) and 800 Hz (red open circles) frequencies for 1 hour, 1 week, 2 weeks, 3 weeks, and 4 weeks. Locomotor activity was recorded for 1 hour following each timepoint and swimming activity was randomly quantified for three zebrafish at seven equally spaced 1-minute periods. The results of the first 1-minute period are not presented due to methodological interference. Data from all six remaining periods were included and pooled together for statistical analyses. Results were analyzed by a two-way ANOVA with duration and frequency of sound exposure as independent factors. Asterisks indicate durations of sound exposure that were statistically significantly different from all other durations of sound exposure.
Figure 2.5. Brightfield images anti-proliferating cell nuclear antigen immunohistochemical staining within the descending octaval nucleus of the zebrafish auditory hindbrain. Immunohistochemical analysis of zebrafish brain 30μm sagittal sections was employed to quantify the number of proliferating cells regionally within hindbrain auditory nuclei. (A) A representative descending octaval nucleus (DON) section demonstrating proliferating cell nuclear antigen (PCNA) immunoreactivity. (B) A representative DON section demonstrating non-specific staining. Scale bars = 100μm.
CHAPTER 3
CONCLUSIONS AND RECOMMENDATIONS

3.1 Summary

The auditory systems of fishes maintain a great propensity for plasticity throughout the lifespan, facilitated by the complex integration of neurophysiological, morphological, molecular, and transcriptional mechanisms initiated in response to intrinsic and environmental factors (e.g. Barcellos et al., 2018; Smith & Monroe, 2016; Forlano et al., 2016; Higgs et al., 2002). Heretofore, functional alterations in auditory circuit sensitivity within the context of acoustic reproductive communication (Forlano et al., 2016) and noise-induced trauma (Smith & Monroe, 2016) and the underpinning mechanisms operating at the level of the auditory periphery have been the primary areas of focus within the study of auditory system plasticity in fishes. While this focus has undoubtably yielded significant advancement, we have yet to understand the contributions of the central auditory system in mediating plasticity in auditory function. Furthermore, the prevailing conservational perspectives (e.g. Cox et al., 2018; Mickle & Higgs, 2017) of research examining the effects of the auditory environment on fishes has resulted in a wealth of knowledge concerning the effects of noise while limiting our understanding of the impacts of non-traumatic acoustic environments on the auditory system and its function in fishes. Through the integrative and comparative study of the effects of various parameters of the non-traumatic auditory environment on the fish central nervous system, we will gain a more holistic understanding of the drivers, underlying mechanisms, and consequences of auditory system plasticity in fishes.

The primary objective of this thesis is to bridge the prevailing gaps in our current understanding of fish auditory system plasticity, that is, to determine the effect of the non-traumatic auditory environment on the central nervous system of fishes. Employing immunohistochemical analysis and a candidate gene transcription profiling approach, Chapter 2 examines the effect of prolonged exposure to continuous tonal stimuli varying in frequency on the regional and global transcriptional response of neuroplasticity-associated genes while also delineating a time course of expression within the zebrafish brain. My results indicate a significant effect of duration and near-significant effect of frequency of prolonged sound exposure on the expression of \( bdnf \) within the zebrafish...
whole brain. The expression of \textit{bdnf} within the zebrafish whole brain across the 4-week sound treatment period manifested an inverted bell curve response, nadiring at 2 weeks post-stimulus onset in response to prolonged exposure to both 800 Hz and 100 Hz treatments. Although a non-significant effect of sound exposure duration, \textit{neurod1} and \textit{atoh1a} exhibited a parallel and inversely parallel temporal pattern of whole brain transcriptional flexibility following long-term exposure to both tone frequencies, respectively. Contrastingly, the regional auditory hindbrain and global expression of \textit{pcna}, a common marker of cellular proliferation in fishes (Grandel et al., 2006), did not exhibit variation as function of either frequency or duration of sound exposure. In light of this disparity between the sound-induced temporal pattern of transcriptional flexibility of genes associated with neuronal connectivity (Dawson et al., 2015; Anomal et al., 2013; Jahan et al., 2010) and cellular addition (Grandel et al., 2006), I speculate that prolonged sound exposure induces connective plasticity rather than sensory experience-induced neurogenesis (Dunlap et al., 2016) within the zebrafish brain. Additionally, zebrafish exhibited differential \textit{bdnf} transcription and swimming behaviour in response to the prolonged 100 Hz and 800 Hz tone exposures. The differential sensitivity of the mechanosensory systems of zebrafishes to these tones and demonstrated transcriptional and behavioural effect of the multisensory 100 Hz tone may implicate a role for multisensory integration, as a consequence of frequency of mechanosensory-evoked behaviour and sensory stimulation, in promoting sound-induced transcriptional flexibility within the zebrafish brain.

A vast body of evidence has established the tremendous flexibility of the fish central nervous system in response to the sensory environment (reviewed in Dunlap, 2016). As noted previously, evidence regarding the effects of the auditory environment on the central nervous system of fishes is limited (Barcellos et al., 2018; Papoutsoglou et al., 2015; Papoutsoglou et al., 2013; Papoutsoglou et al., 2010; Papoutsoglou et al., 2007). The present study provides evidence of sound-induced transcriptional flexibility within the central nervous system of fishes and is the first, to my knowledge, to investigate the effect of prolonged pure tone exposure on brain neuroplasticity-associated transcriptional activity in fishes. This research also contributes to the study of low frequency sound as a multisensory stimulus in fishes (Higgs & Radford, 2016; Higgs &
Radford, 2013) and implicates the lateral line system input as a potential contributing source of central nervous system transcriptional flexibility in “acoustic” contexts.

3.2 Future Directions

We have only just begun to understand the role of the auditory environment in shaping the central nervous system of fishes. As we advance in our pursuit, suggestions for future lines of research considering the findings of the present study may can help to guide our efforts. First, future studies should conduct a similar, more integrative design which encompasses the evaluation of potential acoustic trauma within the auditory periphery. Curiously, the findings suggesting a downregulation of whole brain $bdnf$ expression in response to 2 weeks of tonal acoustic experience presented within this thesis are incongruent that of pervious research demonstrating increased transcription of $bdnf$ following a 15-day classical music treatment period (Barcellos et al., 2018). Extending the objective of the current study and investigating the contributions of various parameters of sound stimuli to transcriptional flexibility is imperative as long-term exposure to acoustic parameters other than frequency and duration may differentially impact the central auditory system (e.g. complexity, amplitude). However, this apparent contradiction may be attributed to acoustic overexposure. Future morphological analysis for saccular hair cell damage and electrophysiological measurement of hearing sensitivity conducted post-prolonged tone exposure may explain this disparity of findings. Furthermore, investigations regarding the impact of acoustic trauma within the central auditory system are necessary to gain a more thorough understanding of the consequences of anthropogenic noise on fishes.

Ideally, future investigations will aim to understand the mechanisms underlying acoustic experience-induced central auditory system plasticity in fishes through direct manipulations. Pressing questions inspired by this study include: What is the functional significance of $bdnf$ following acoustic experience? What does the expression of $bdnf$ vary as a function of sound exposure duration? Why is the underlying cause of the differential transcription plasticity exhibited between zebrafish exposed to the 100 Hz and 800 Hz tones? Does lateral line system stimulation by low frequency sound contribute to central auditory system plasticity? A large proportion of these queries can be addressed through direct manipulations of the sensory systems of fishes. For example,
through blocking BDNF function, Anomal et al. (2013) discovered the role of BDNF in facilitating pure tone experience-dependent tonotopic map plasticity within the rat primary auditory cortex. Similar studies conducted in fishes may aid in the delineation of the mechanisms contributing to auditory system plasticity. Similarly, the contributions of the lateral line system stimulation to the transcriptional flexibility exhibited within the zebrafish brain in response to the 100 Hz tone could be addressed through an extension of the present study in which in the zebrafish lateral line is chemically ablated. This would permit the isolated study of the mechanosensory effects of sound detected solely by the inner ear on the transcriptional flexibility of the central nervous system. Thus, through the direct manipulation of the acoustic environment and sensory systems of a diverse array of fishes, we will contribute to the development of a more well-rounded, integrative understanding auditory system plasticity in fishes.
References


APPENDIX A: GRAPHS

A  

B  

C  

D  

118
Figure A.1. Normalized transcript abundance of candidate neuroplasticity genes within the zebrafish (Danio rerio) brain in response to long-term acoustic pure tone exposure. Normalized whole zebrafish brain transcription (ΔC\textsubscript{q}) of targeted neuroplasticity-associated genes (A) brain-derived neurotrophic factor (bdnf), (B) (neuronal differentiation factor 1 (neurod1), (C) atonal homolog 1a (atoh1a), and (D) proliferating cell nuclear antigen (pcna) in response to long-term sound exposure 100 Hz (solid blue circles) or 800 Hz (open red circles) at a sound pressure level of 140 dB (re 1 μPa) or silence (open purple squares) for durations of 1 hour, 1 week, 2 weeks, 3 weeks, or 4 weeks. Expression levels are normalized to an index provided by the geometric mean of elongation factor-1a (ef1a) and β-actin expression and plotted as individual values. Individual sample values presented here were used in the calculation of relative whole brain transcription (ΔΔC\textsubscript{q}). Horizontal bars and associated error bars denote the mean ± standard error of the mean. The solid blue triangle denotes an outlier existed within the 3-week 100 Hz exposure group which was excluded from all statistical analyses.
Figure A.2. Relative mRNA expression levels of targeted neuroplasticity-associated genes within the zebrafish (Danio rerio) whole brain following prolonged acoustic tonal exposure. Relative whole brain transcription (ΔΔC_q) of (A) brain-derived neurotrophic factor (bdnf), (B) neural differentiation factor 1 (neurod1), (C) atonal homolog 1a (atoh1a), and (D) proliferating cell nuclear antigen (pcna) following 1 hour, 2 weeks, 3 weeks, or 4 weeks exposure to a 100 Hz (solid blue circles) or 800 Hz (open red circles) tone. Results are reported relative to the mean normalized whole brain transcript abundance (ΔC_q) of the silent controls within the respective silent control group of matched duration. Individual ΔΔC_q values reported were employed for the calculation of gene expression fold change. Values are presented as individual results with horizontal bars and associated error bars to denote the mean ± standard error of the mean expression of the respective treatment group. A solid blue triangle denotes an outlier within the 100 Hz exposure for 3 weeks condition which was exempt from statistical analyses.
Figure A.3. Fold change of neuroplasticity-associated gene transcription within the zebrafish (Danio rerio) brain following long-term acoustic pure tone exposure. The transcriptional responses of (A) brain-derived neurotrophic factor (bdnf), (B) neural differentiation factor 1 (neurod1), (C) atonal homolog 1a (atoh1a), and (D) proliferating cell nuclear antigen (pcna) within the zebrafish whole brain to 1-hour, 1-week, 2-week, 3-week, and 4-week 100 Hz (solid blue circles) and 800 Hz (open red circles) pure tone acoustic exposures are reported here as individual fold change values with group means ± standard error of the mean expressed as horizontal lines and associated error bars. Fold change was calculated using the $2^{-\Delta\Delta C_q}$ method (Livak & Schmittgen, 2001); however, the theoretical polymerase chain reaction (PCR) efficiency of 2 within this formula was replaced by the gene-specific LinRegPCR-estimated PCR efficiency. All PCR efficiencies used in the calculation of fold change are reported in Table 2.1. Fold change values of 1 suggest non-differential expression from silent controls (Bergemann & Wilson, 2011), while fold change values below and above 1 suggests the gene was differentially expressed, that is, downregulated or upregulated, respectively, in sound exposed fish relative to silent controls. Although not included in statistical analyses, we report an outlier denoted by a solid blue triangle.
APPENDIX B: PROTOCOLS

**RNA Extraction Protocol**

1. Fill a 2 mL microcentrifuge tube to the 0.5 mL mark with standard glass beads and add the sample.
2. Add 0.75 mL of Trizol to the microcentrifuge tube.
3. Homogenize the sample for three 1-minute intervals, spaced 1 minute apart.
4. Collect the supernatant and centrifuge at 13,000 rpm at 4°C for 15 minutes.
5. Collect the supernatant and add 0.2 mL of chloroform. Vortex the mixture then centrifuge at 13,000 rpm at 4°C for 15 minutes.
6. Collect 300 μL of the top, clear layer.
7. Add 300 μL isopropanol and vortex the mixture.
8. Allow sample to mix on an orbital shaker for 30 minutes at 25°C.
9. Centrifuge at 13,000 rpm at 4°C for 15 minutes.
10. Isolate the pellet.
11. Add 0.5 mL of cold 85% ethanol and centrifuge at 13,000 rpm at 4°C for 3 minutes. Isolate pellet. Repeat step once.
13. Invert the Eppendorf tube on a bench top covered with low-lint tissue. Allow the pellet to dry for approximately 30 minutes.
14. Resuspend the pellet in 30 μL of ddH₂O and 3 μL RNase inhibitor.
15. Add 3 μL of DNase 1 reaction buffer and 1 uL of DNase.
16. Incubate at 37°C for 10 minutes.
17. Add 1 μL 50 mM EDTA.
18. Incubate at 75°C for 10 minutes.
19. Store RNA sample at -80°C.
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